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(54) **DOCK-AND-LOCK (DNL) VACCINES FOR CANCER THERAPY**

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(57) **ABSTRACT**

The present invention concerns methods and compositions for forming anti-cancer vaccine DNL complexes using dock-and-lock technology. In preferred embodiments, the anti-cancer vaccine DNL complex comprises an antibody moiety that binds to dendritic cells, such as an anti-CD74 antibody or antigen-binding fragment thereof, attached to an AD (anchoring domain) moiety and a xenoantigen, such as CD20, attached to a DDD (dimerization and docking domain) moiety, wherein two copies of the DDD moiety form a dimer that binds to the AD moiety, resulting in the formation of the DNL complex. The anti-cancer vaccine DNL complex is capable of inducing an immune response against xenoantigen expressing cancer cells, such as $CD138^{neg}CD20^+$ MM stem cells, and inducing apoptosis of and inhibiting the growth of or eliminating the cancer cells.

9 Claims, 16 Drawing Sheets

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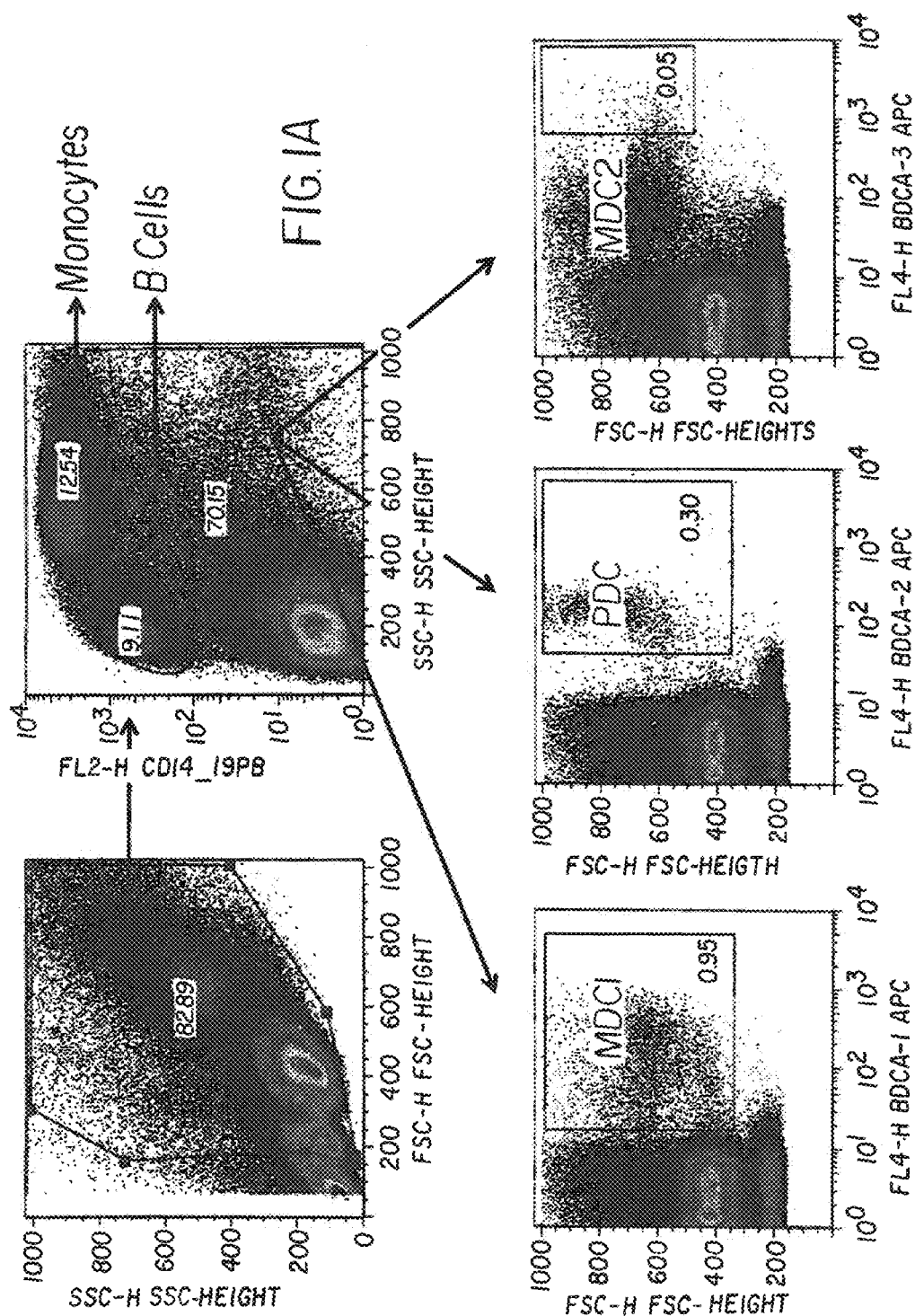
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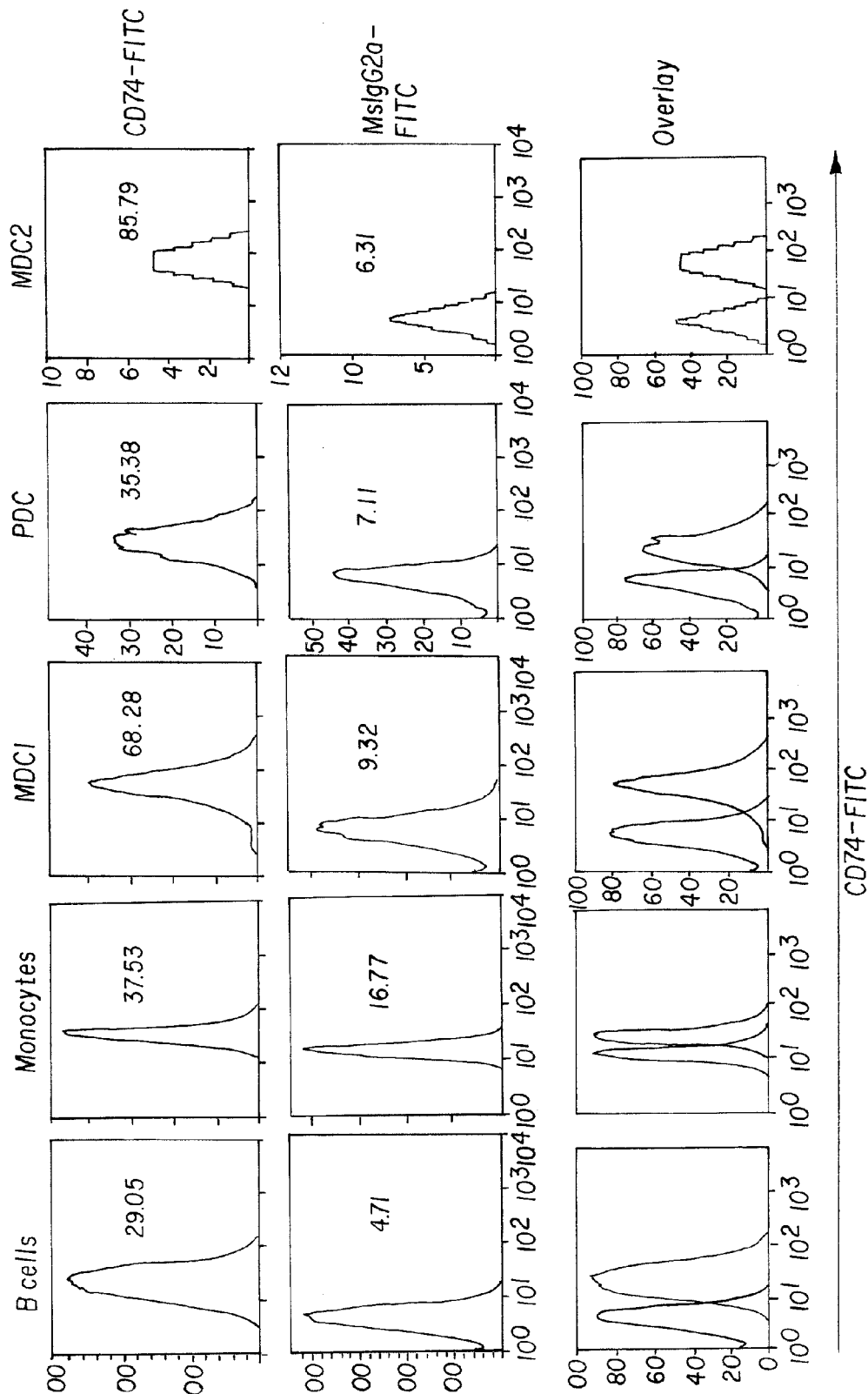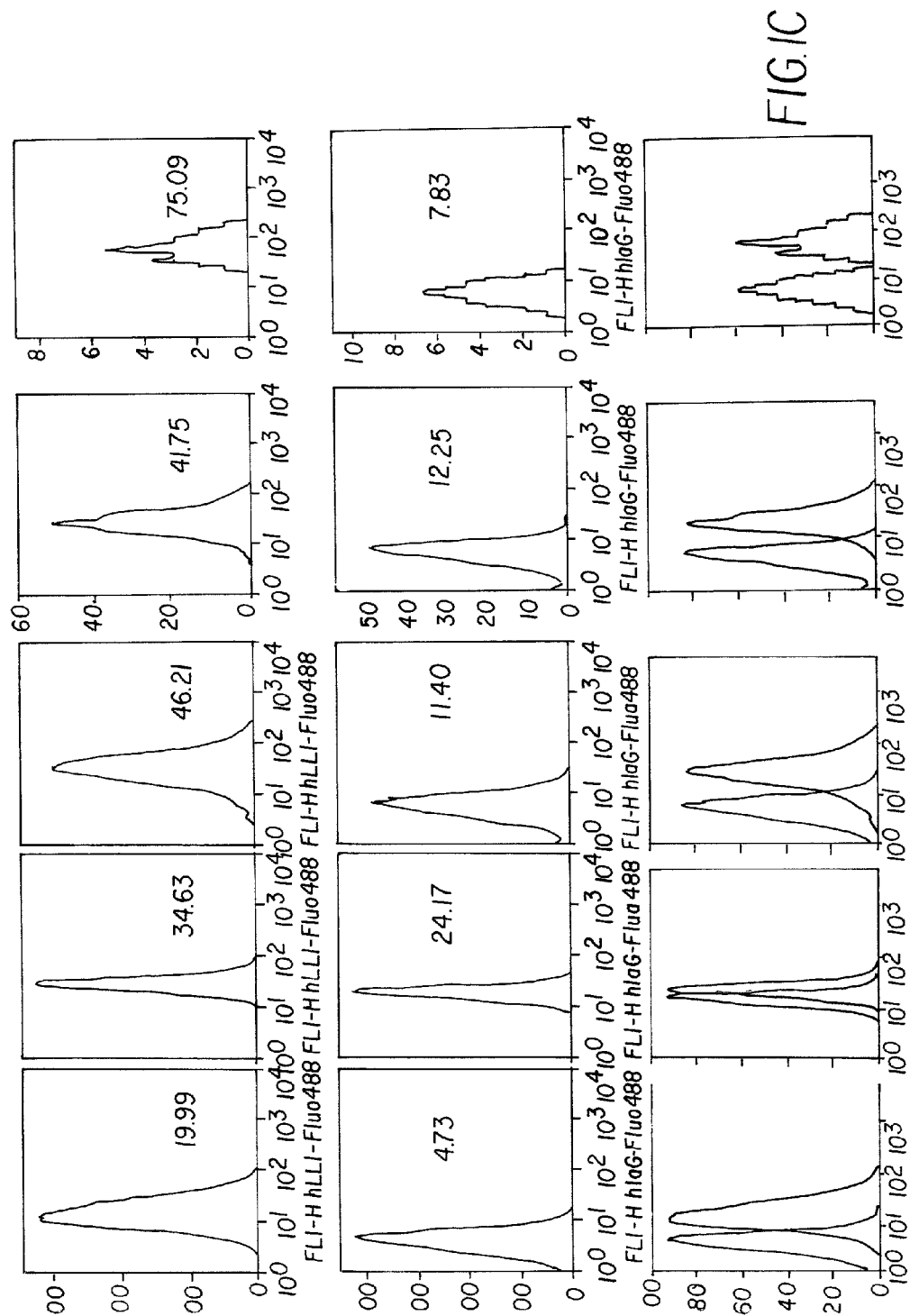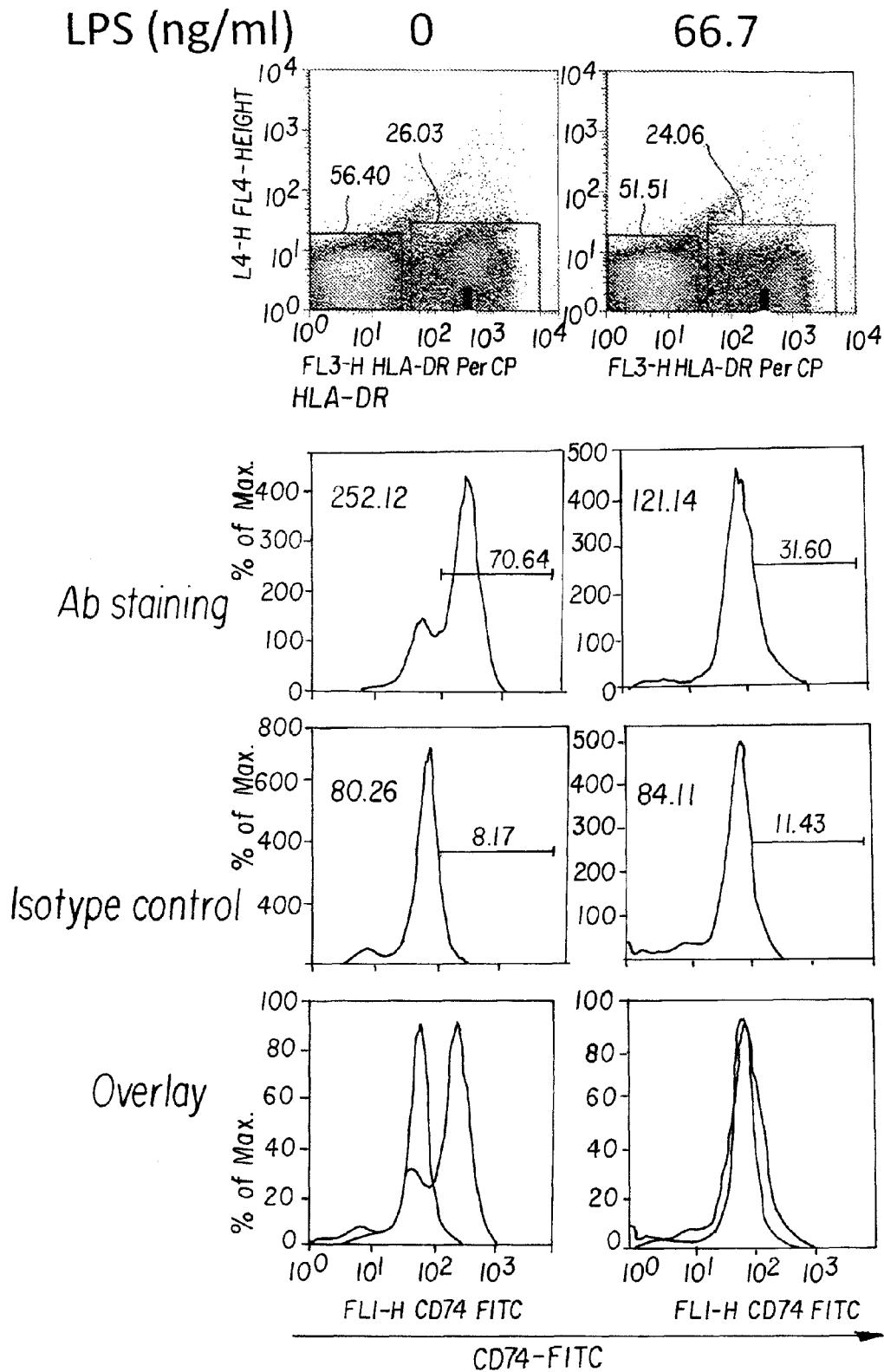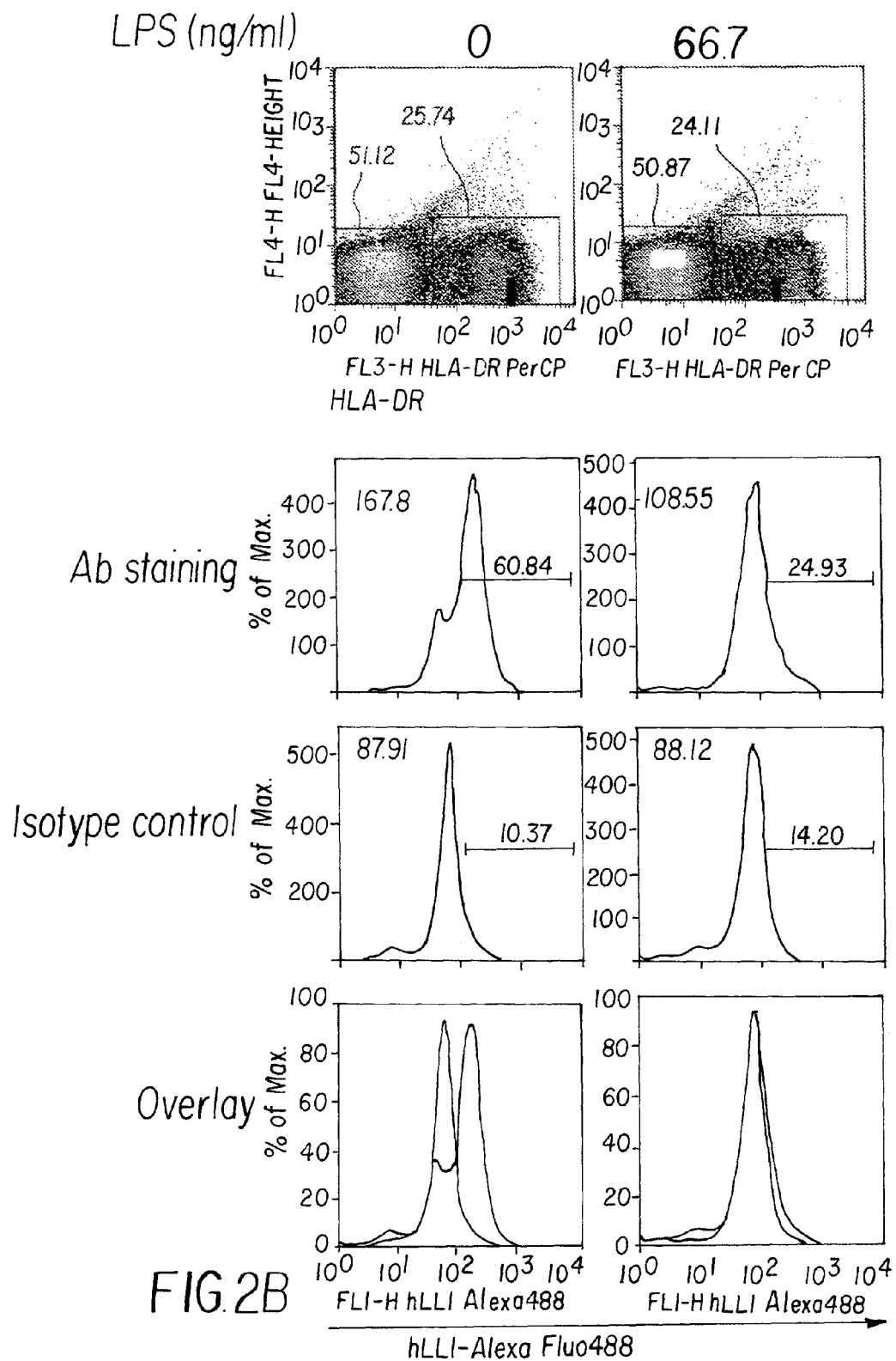
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FIG.1A
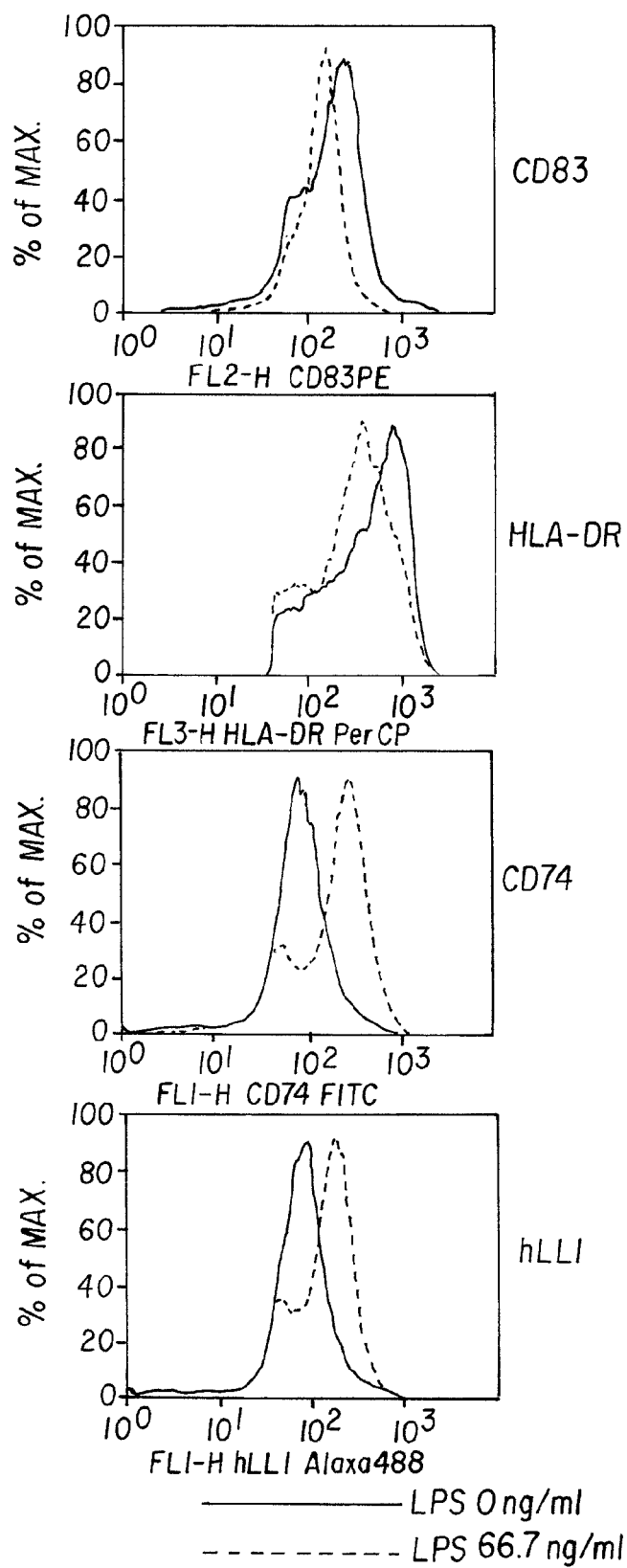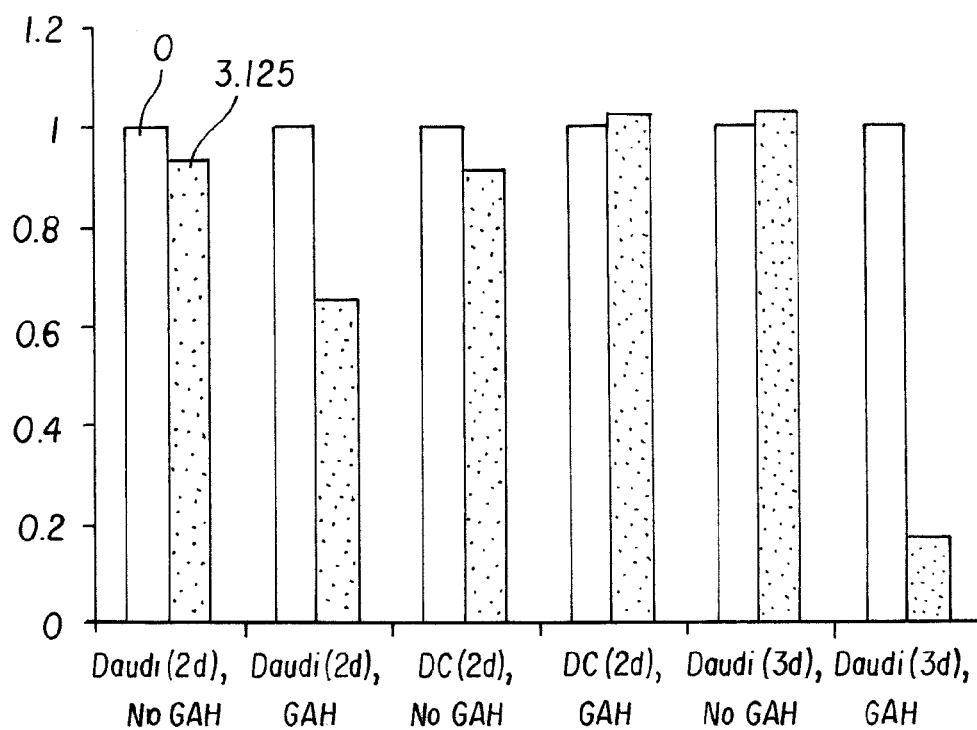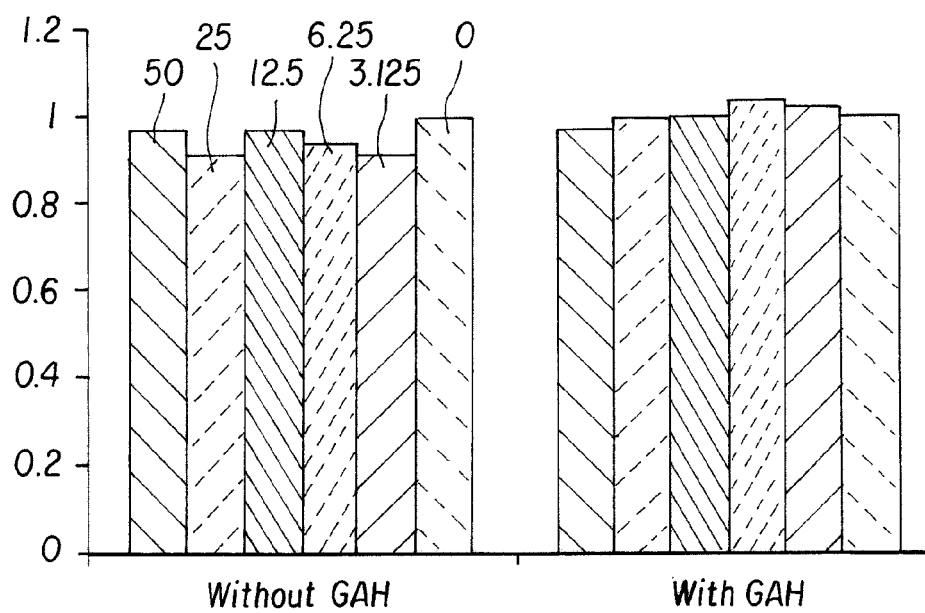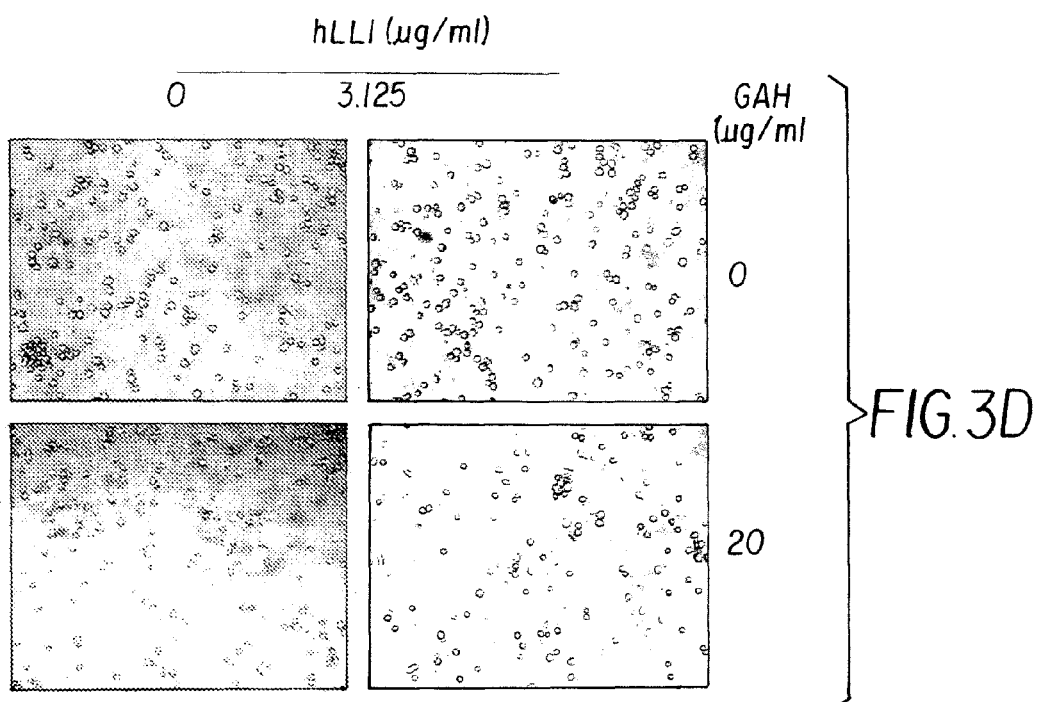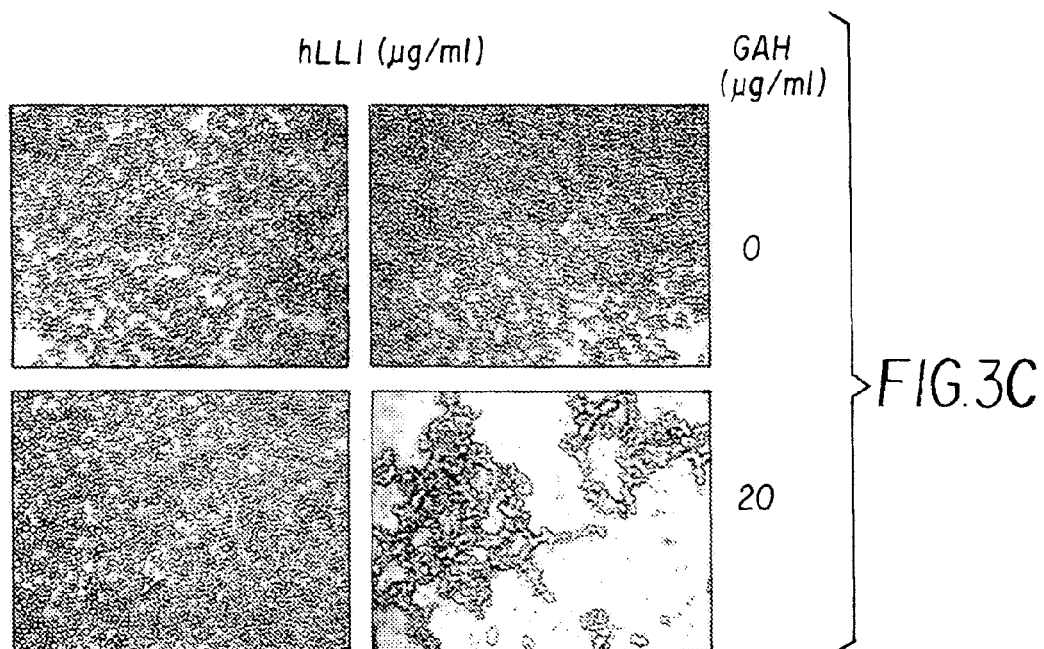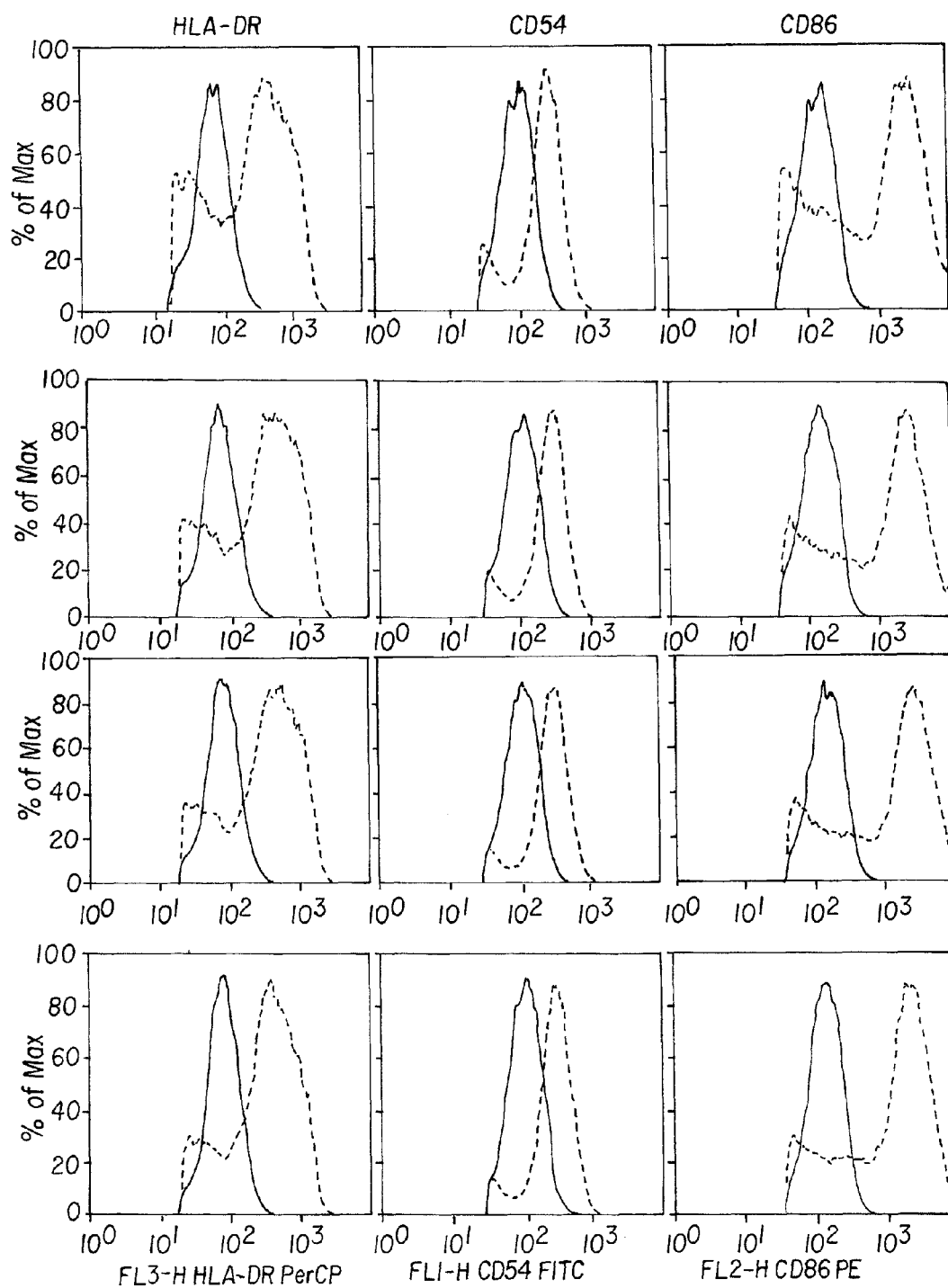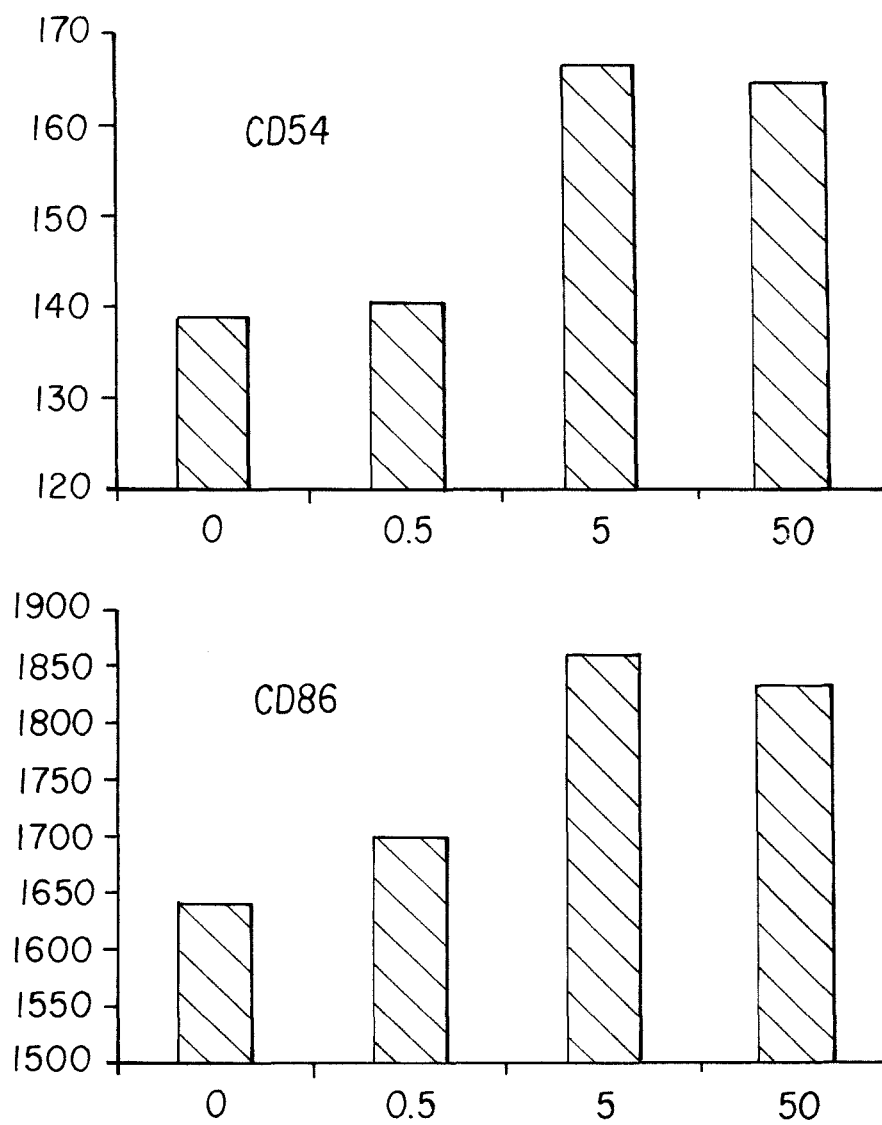
FIG. IB

FIG.IC

FIG. 2A

FIG.2B

FIG. 2C

——— LPS 0 ng/ml

- - - - - - - LPS 66.7 ng/ml

FIG. 3A

FIG. 3B

hLLI (μg/ml)   GAH
                              (μg/ml)

0

20

FIG.3C

hLLI (μg/ml)

0   3.125   GAH
                              (μg/ml

0

20

FIG.3D

FIG. 4A

FIG. 4B

FIG. 5A1	FIG. 5A2

FIG. 5A

FIG. 5B1	FIG. 5B2

FIG. 5B

FIG. 5AI

FIG. 5A2

Total T cells
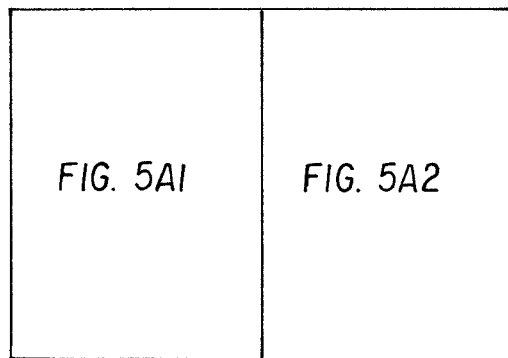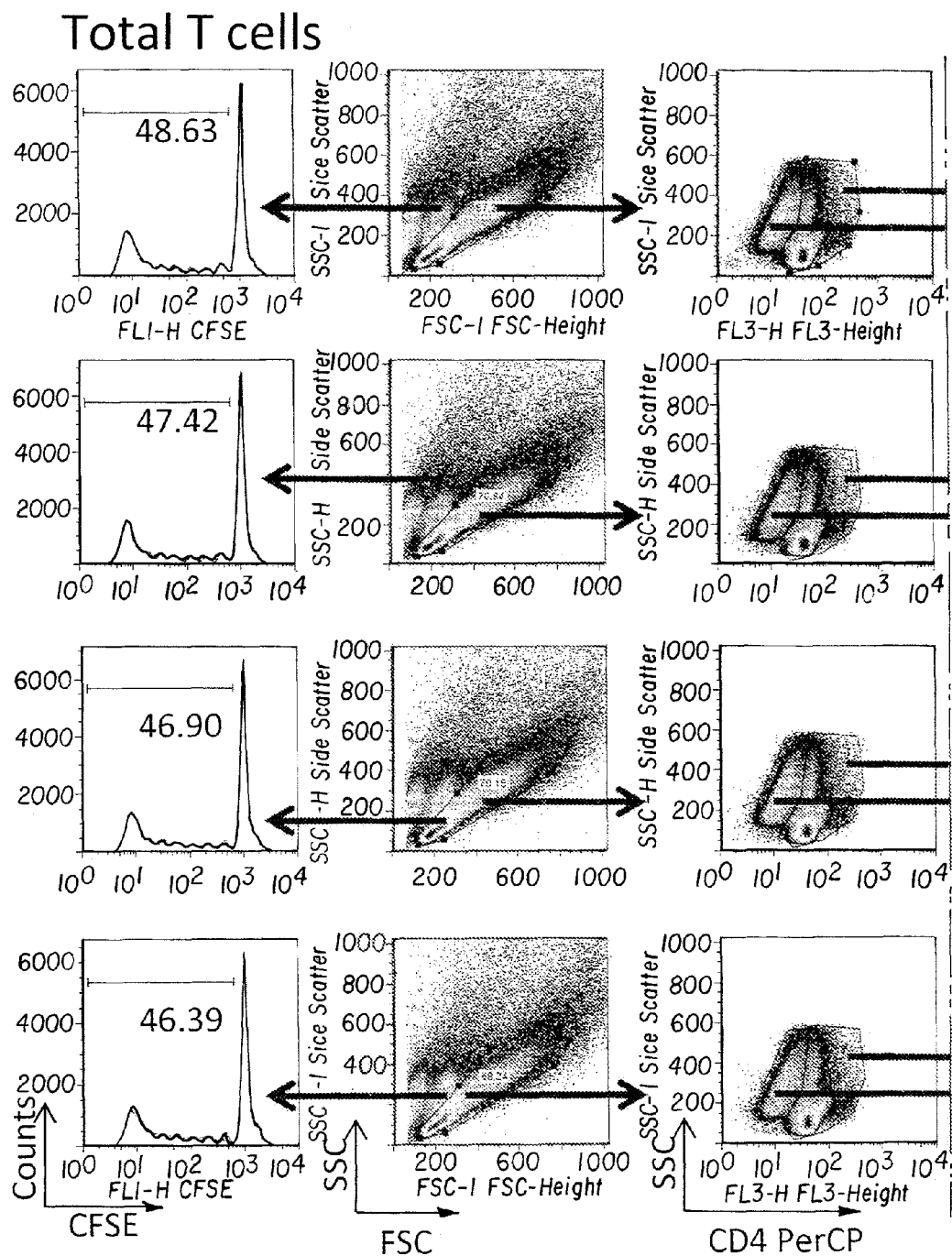
FIG. 5BI

FIG. 5B2

FIG. 6

DOCK-AND-LOCK (DNL) VACCINES FOR CANCER THERAPY

RELATED APPLICATIONS

This application is a divisional of U.S. patent application Ser. No. 12/544,476, filed Aug. 20, 2009, which was a continuation-in-part of U.S. patent application Ser. No. 12/396,605 (now issued U.S. Pat. No. 7,858,070), filed Mar. 3, 2009, which was a divisional of U.S. patent application Ser. No. 11/633,729 (now issued U.S. Pat. No. 7,527,787), filed Dec. 5, 2006, which was a continuation-in-part of U.S. patent application Ser. No. 11/389,358 (now issued U.S. Pat. No. 7,550,143), filed Mar. 24, 2006; Ser. No. 11/391,584 (now issued U.S. Pat. No. 7,521,056), filed Mar. 28, 2006, and Ser. No. 11/478,021 (now issued U.S. Pat. No. 7,534, 866), filed Jun. 29, 2006, and which claimed the benefit under 35 U.S.C. 119(e) of provisional U.S. Patent Application Nos. 60/782,332, filed Mar. 14, 2005; 60/728,292, filed Oct. 19, 2005; 60/751,196, filed Dec. 16, 2005; and No. 60/864,530, filed Nov. 6, 2006. This application c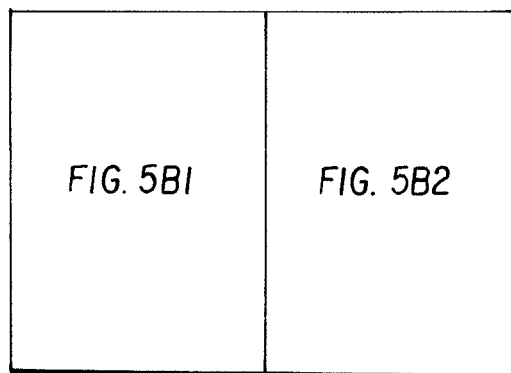laims the benefit under 35 U.S.C. 119(e) of U.S. Provisional Patent Application Ser. No. 61/090,487, filed Aug. 20, 2008. The entire text of each priority application is incorporated herein by reference.

BACKGROUND

1. Field of the Invention

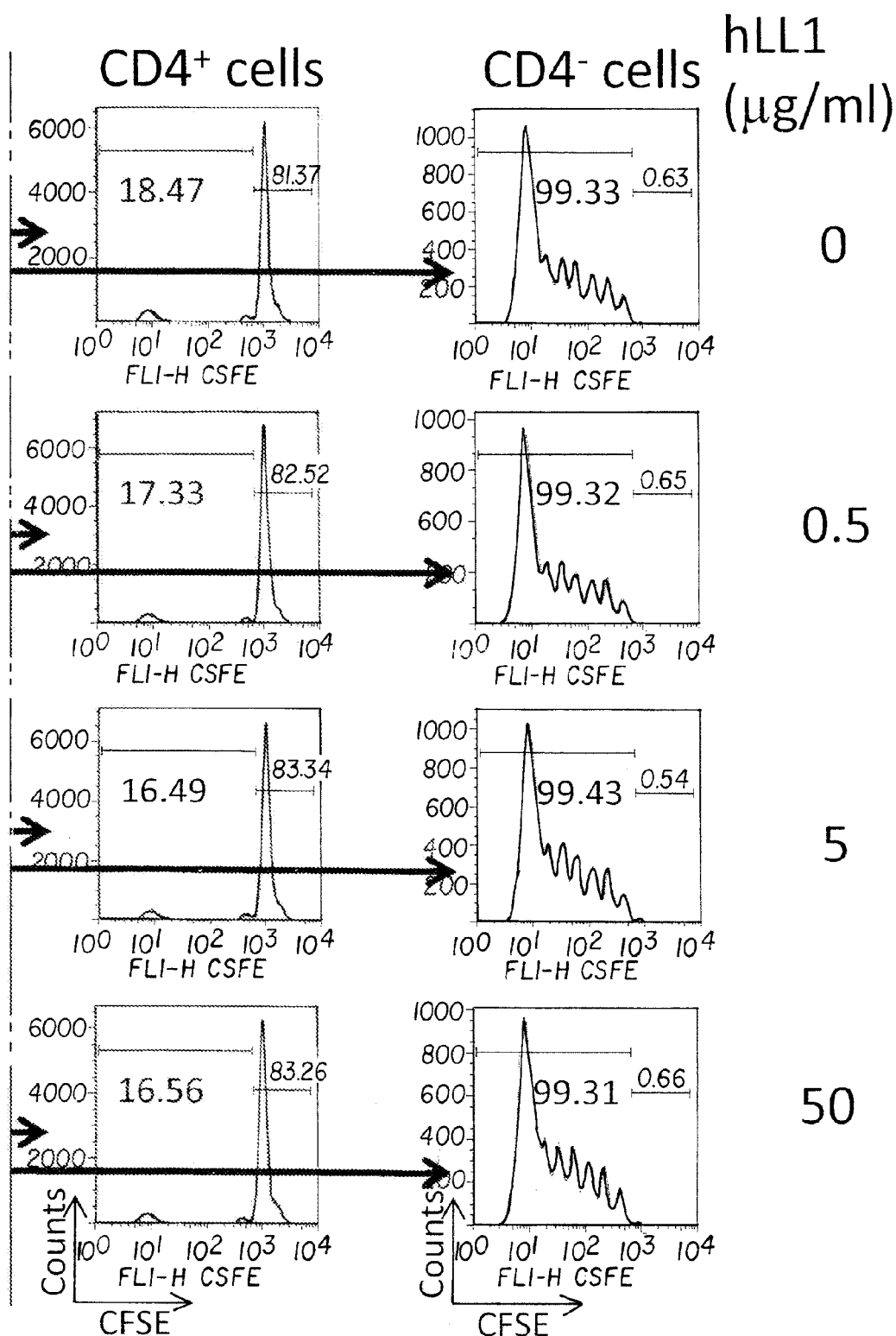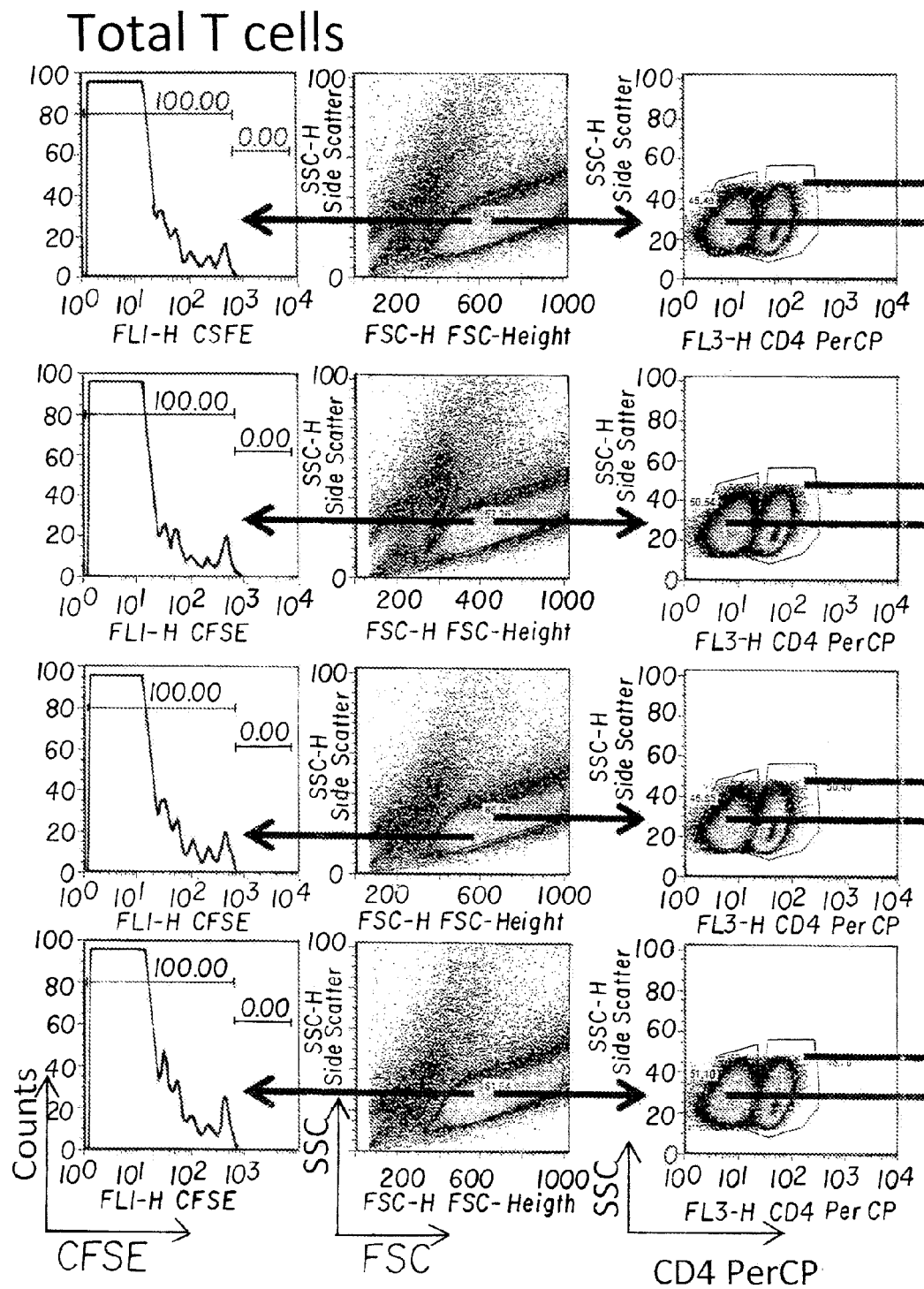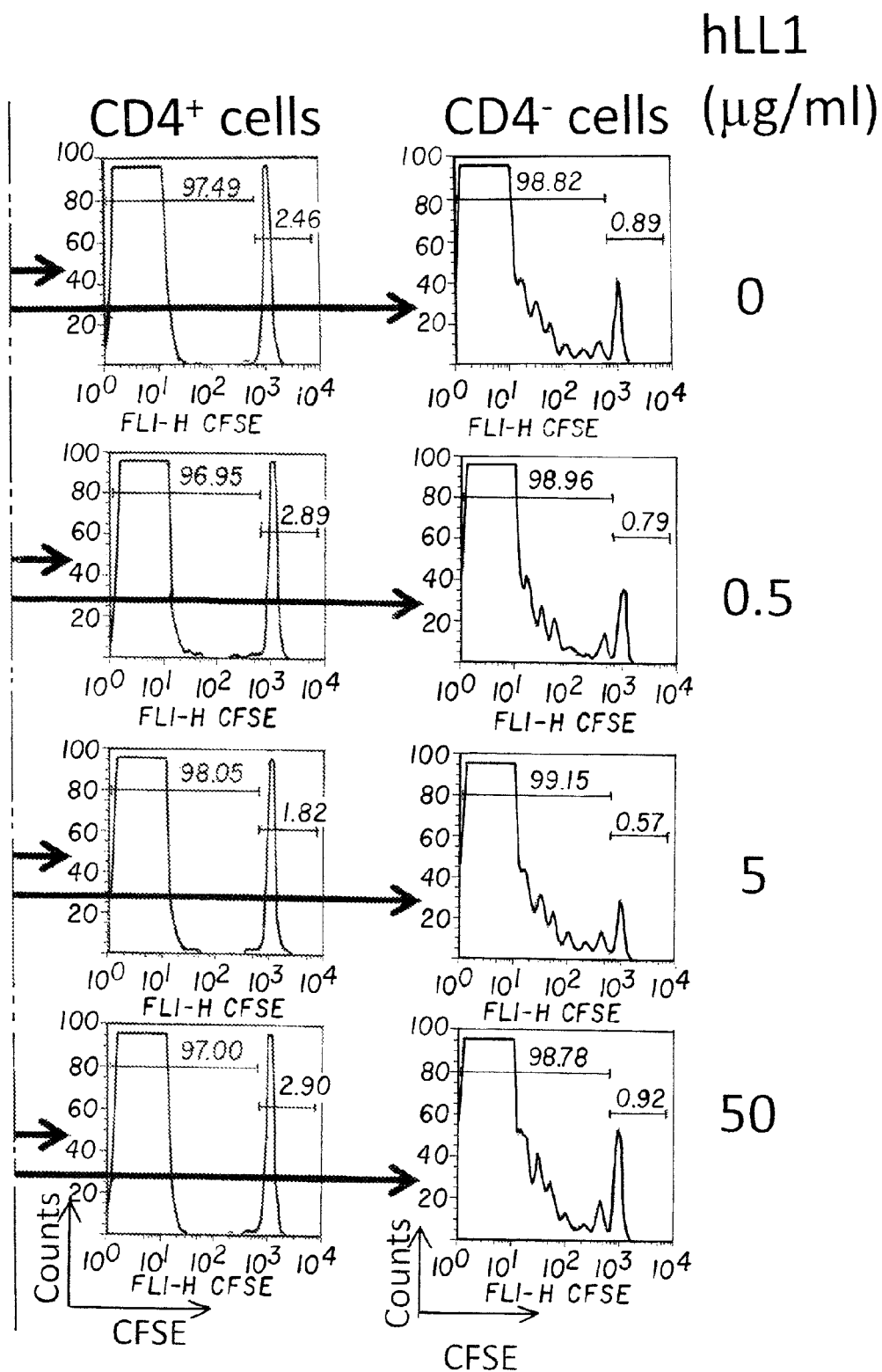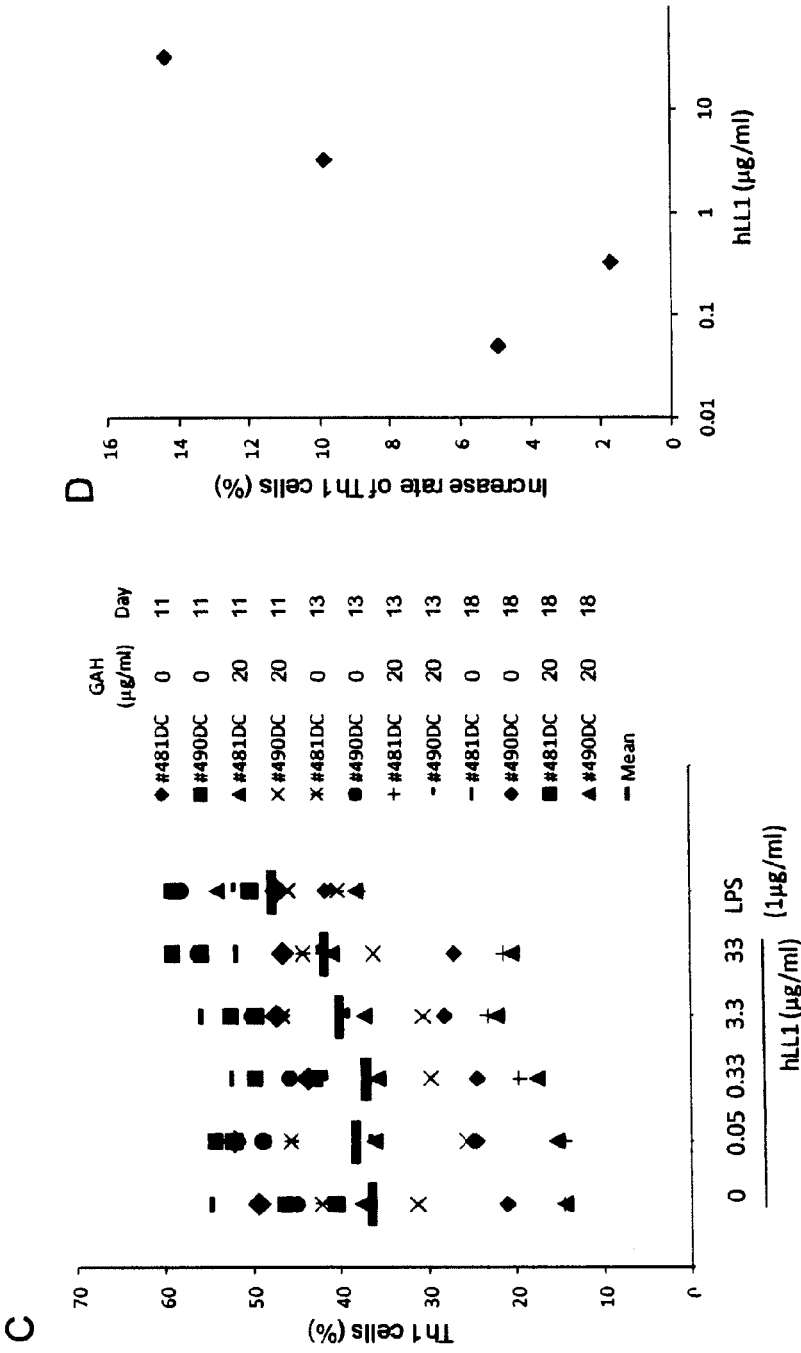The present invention relates to the design and generation of dendritic cell-based, in vivo antigen targeting vaccines for therapy of cancer, such as multiple myeloma. In preferred embodiments the vaccines are generated by the dock-and-lock (DNL) method, in which effector moieties are attached to anchoring domain (AD) derived from AKAP proteins and dimerization and docking domain (DDD) moieties derived from protein kinase A (PKA). DNL complexes are generated when DDD moieties spontaneously dimerize and bind to an AD moiety, resulting in a complex with a 2:1 stoichiometry between DDD and AD-linked effectors. In more preferred embodiments, the effector moieties comprise a humanized anti-CD74 antibody and a tumor-associated xenoantigen, such as a CD20 xenoantigen. In most preferred embodiments, the anti-CD74 antibody is an hLL1 antibody. The DNL constructs are of use for preparation of pharmaceutical compositions, for generation of vaccines against cancers, such as multiple myeloma (MM), and for induction of an immune response against tumor antigen-expressing cells, such as CD20 positive cancer cells in patients with multiple myeloma or other CD20-expressing cancers.

2. Related Art

Multiple myeloma (MM) is a hematological malignancy characterized by clonal proliferation of neoplastic plasma cells in the bone marrow. Although responsive to many chemotherapeutic agents, MM remains largely incurable and the majority of patients ultimately relapse, due to the existence of a minor population of MM cancer stem cells that survive standard or high-dose chemotherapy and are resistant to chemotherapeutic drugs (Reece et al., Leuk Lymphoma, 2008, 49:1470-85). This small number of MM cancer stem cells constitutes the minimal residual disease and causes relapse, eventually leading to the failure of all treatments. Thus, eradication of MM cancer stem cells may offer a long-term control or even cure of MM.

Recently, a small population of clonotypic B cells, that do not express the characteristic plasma cell surface antigen CD138 but do express the B cell antigen CD20, was identified from both MM cell lines and primary bone marrow of MM patients (Matsui et al., Blood 2004, 103:2332-6). This small population of cells is resistant to multiple clinical anti-myeloma drugs and is capable of clonogenic growth in vitro (Matsui et al., Blood 2004, 103:2332-6; Matsui et al., Cancer Res. 2008, 68:190-7) and in a 3-D culture model (Kirshner et al., Blood 2008, 112:2935-45), and is capable of differentiation into MM cells in vitro and in engrafted NOD/SCID mice during both primary and secondary transplantation (Matsui et al., Cancer Res. 2008, 68:190-7). It has thus been suggested that these CD138^{neg}CD20$^+$ cells represent the putative multiple myeloma cancer stem cells (Huff and Matsui, J Clin Oncol. 2008, 26:2895-900).

Like other cancer stem cells, MM cancer stem cells are refractory to multiple chemotherapeutic drugs and responsible for tumor re-growth and relapse (Huff and Matsui, J Clin Oncol. 2008, 26:2895-900; Yang and Chang, Cancer Invest. 2008, 26:741-55). Strategies and approaches that could selectively target and eradicate cancer stem cells, such as MM stem cells, are needed. Due to the multiple drug resistance in cancer stem cells, immunotherapy and vaccination may offer a potential modality to eradicate these cells, particularly after standard therapies and/or stem cell transplantation, the time when tumor load is greatly reduced. A need exists for effective compositions and methods of immunotherapy and vaccination targeted to treatment of multiple myeloma, particularly those capable of inducing an immune response against and inhibiting or eradicating MM cancer stem cells. A further need exists for effective compositions and methods of immunotherapy and vaccination targeted to treatment of cancers in general.

SUMMARY OF THE INVENTION

The present invention discloses methods and compositions for vaccines against cancer stem cells, such as MM stem cells, that are prepared using the Dock-and-Lock (DNL) method (Chang et al., 2007, Clin Cancer Res 13:5586s-91s). The DNL technique has been used to generate a variety of stable and defined complexes suitable for in vivo applications. In preferred embodiments, the DNL complexes comprise an anti-CD74 antibody or antigen binding fragment thereof, such as the hLL1 antibody, attached to a dimerization and docking domain (DDD) or anchor domain (AD) moiety. The DDD moieties spontaneously dimerize and each DDD dimer binds to an AD moiety. In more preferred embodiments, a complementary AD or DDD moiety is attached to a CD20 xenoantigen, as described in further detail below, resulting in formation of DNL complexes comprising anti-CD74 moieties and CD20 xenoantigen moieties. However, the skilled artisan will realize that depending on the cancer, a different xenoantigen and/or antibody or antibody fragment may be utilized. The antibody component directs the DNL complex to antigen presenting cells (APCs), such as dendritic cells (DCs), while the xenoantigen component is processed to invoke an immune response against cells expressing the target antigen.

Various types of DNL complexes with different structures and different ratios of target antigen (e.g., CD20) to antibody or antibody fragment may be constructed and used within the scope of the claimed methods and compositions, such as those disclosed in U.S. Pat. No. 7,550,143 (incorporated herein by reference from Col. 28, line 30 through Col. 44, line 28); U.S. Pat. No. 7,521,056 (incorporated herein by reference from Col. 58, line 1 through Col. Col. 84, line 45); U.S. Pat. No. 7,534,866 (incorporated herein by reference

from Col. 31, line 1 through Col. 36, line 38); U.S. Pat. No. 7,527,787 (incorporated herein by reference from Col. 61, line 51 through Col. 94, line 65) and U.S. Patent Appl. Publ. No. 2009/006082 (incorporated herein by reference from paragraph [0035] through paragraph [0097]). DNL complexes comprised of trimeric, tetrameric, pentameric, hexameric and other structures have been reported in the above-cited issued patents.

In most preferred embodiments, the anti-cancer vaccine DNL construct comprises a humanized, or chimeric LL1 anti-CD74 antibody or antigen-binding fragment thereof comprising the light chain variable complementarity-determining region (CDR) sequences CDR1 (RSSQS-LVHRNGNTYLH; SEQ ID NO:1), CDR2 (TVSNRFS; SEQ ID NO:2), and CDR3 (SQSSHVPPT; SEQ ID NO:3) and the heavy chain variable region CDR sequences CDR1 (NYGVN; SEQ ID NO:4), CDR2 (WINPNTGEPTFDDD-FKG; SEQ ID NO:5), and CDR3 (SRGKNEAWFAY; SEQ ID NO:6). A humanized LL1 (hLL1) anti-CD74 antibody suitable for use in the claimed DNL complexes is disclosed in U.S. Pat. No. 7,312,318, incorporated herein by reference from Col. 35, line 1 through Col. 42, line 27 and FIG. 1 through FIG. 4. Alternatively, other anti-CD74 antibodies or antibodies against other APC- or DC-associated antigens may be utilized.

The sequences of various CD20 xenoantigens suitable for use in the anti-cancer vaccine DNL complex are known in the art, such as the murine CD20 sequence (SEQ ID NO:7). Other CD20 amino acid sequences of potential use are readily available to the skilled artisan through such well-known public databases as the NCBI protein database (see, e.g., NCBI Accession Nos. NP 031667; P19437; AAA37394; BAE47068; ABA29631; BAD77809). Although the murine CD20 sequence is recited herein, the skilled artisan will realize that CD20 amino acid sequences are known and readily available from a wide variety of species and can be incorporated into the anti-cancer vaccine DNL complex. However, the skilled artisan will realize that other tumor-associated antigens (TAAs) are known in the art and may be utilized in the DNL complexes to induce an immune response against tumors expressing different TAAs. Known TAAs of potential use include, but are not limited to, carbonic anhydrase IX, alpha-fetoprotein, .alpha.-actinin-4, A3, antigen specific for A33 antibody, ART-4, B7, Ba 733, BAGE, BrE3-antigen, CA125, CAMEL, CAP-1, CASP-8/m, CCL19, CCL21, CD1, CD1a, CD2, CD3, CD4, CD5, CD8, CD11A, CD14, CD15, CD16, CD18, CD19, CD20, CD21, CD22, CD23, CD25, CD29, CD30, CD32b, CD33, CD37, CD38, CD40, CD40L, CD45, CD46, CD52, CD54, CD55, CD59, CD64, CD66a-e, CD67, CD70, CD74, CD79a, CD80, CD83, CD95, CD126, CD133, CD138, CD147, CD154, CDC27, CDK-4/m, CDKN2A, colon-specific antigen-p (CSAp), CEA (CEACAM5), CEACAM6, DAM, EGFR, EGFRvIII, EGP-1, EGP-2, ELF2-M, Ep-CAM, Flt-1, Flt-3, folate receptor, G250 antigen, GAGE, gp100, GROB, HLA-DR, HM1.24, human chorionic gonadotropin (HCG) and its subunits, HER2/neu, HMGB-1, hypoxia inducible factor (HIF-1), HSP70-2M, HST-2, Ia, IGF-1R, IFN-.gamma., IFN-.alpha., IFN-.beta., IL-2, IL-4R, IL-6R, IL-13R, IL-15R, IL-17R, IL-18R, IL-6, IL-8, IL-12, IL-15, IL-17, IL-18, IL-25, insulin growth factor-1 (IGF-1), KC4-antigen, KS-1-antigen, KS1-4, Le-Y, LDR/FUT, macrophage migration inhibitory factor (MIF), MAGE, MAGE-3, MART-1, MART-2, NY-ESO-1, TRAG-3, mCRP, MCP-1, MIP-1A, MIP-1B, MIF, MUC1, MUC2, MUC3, MUC4, MUM-1/2, MUM-3, NCA66, NCA95, NCA90, antigen specific for PAM-4 antibody, placental growth factor, p53,

prostatic acid phosphatase, PSA, PRAME, PSMA, P1GF, ILGF, ILGF-1R, IL-6, IL-25, RS5, RANTES, T101, SAGE, 5100, survivin, survivin-2B, TAC, TAG-72, tenascin, TRAIL receptors, TNF-.alpha., Tn antigen, Thomson-Frie-denreich antigens, tumor necrosis antigens, VEGFR, ED-B fibronectin, WT-1, 17-1A-antigen, complement factors C3, C3a, C3b, C5a, C5, an angiogenesis marker, bcl-2, bcl-6, Kras, cMET, an oncogene marker and an oncogene product (see, e.g., Sensi et al., Clin Cancer Res 2006, 12:5023-32; Parmiani et al., J Immunol 2007, 178:1975-79; Novellino et al. Cancer Immunol Immunother 2005, 54:187-207). Xeno-antigen amino acid sequences, such as murine protein amino acid sequences, may be readily obtained from public databases, such as the NCBI protein database.

The skilled artisan will further realize that other known antibodies or antigen-binding fragments thereof may potentially be incorporated into the anti-cancer vaccine DNL constructs. In preferred embodiments, the antibody binds to an antigen expressed by APCs, more preferably dendritic cells. A variety of antigens associated with dendritic cells are known in the art, including but not limited to CD209 (DC-SIGN), CD34, CD74, CD205, TLR 2 (toll-like receptor 2), TLR 4, TLR 7, TLR 9, BDCA-2, BDCA-3, BDCA-4, and HLA-DR. In preferred embodiments, the target antigen is CD74. However, other types of target antigen are known to be associated with dendritic cells and anti-cancer vaccine DNL constructs incorporating antibodies that target any such alternative antigen may be utilized in the claimed methods and compositions. In some embodiments, the anti-cancer vaccine DNL constructs may comprise an anti-CD74 antibody or antigen-binding fragment thereof and another anti-dendritic cell antibody or fragment. Exemplary antibodies that may be utilized in the anti-cancer vaccine DNL constructs include, but are not limited to, hLL1 (anti-CD74, U.S. Pat. No. 7,312,318) and hL243 (anti-HLA-DR, U.S. patent application Ser. No. 11/368,296) the Examples section of each incorporated herein by reference.

The use of chimeric antibodies is preferred because they possess human antibody constant region sequences and therefore do not elicit as strong a human anti-mouse antibody (HAMA) response as murine antibodies. The use of humanized antibodies is even more preferred, in order to further reduce the possibility of inducing a HAMA reaction. As discussed below, techniques for humanization of murine antibodies by replacing murine framework and constant region sequences with corresponding human antibody framework and constant region sequences are well known in the art and have been applied to numerous murine anti-cancer antibodies. Antibody humanization may also involve the substitution of one or more human framework amino acid residues with the corresponding residues from the parent murine framework region sequences. As also discussed below, techniques for production of human antibodies are also well known and such antibodies may be incorporated into the subject anti-cancer vaccine constructs.

In certain embodiments, the anti-cancer vaccine DNL constructs may be administered in combination with at least one therapeutic agent administered before, simultaneously with or after the anti-cancer vaccine construct. In preferred embodiments, the therapeutic agent is administered before the anti-cancer vaccine. However, in alternative embodiments, the therapeutic agent may be co-administered with or even conjugated to the DNL construct. Any therapeutic agent known in the art, as discussed in more detail below, may be utilized in conjunction with an anti-cancer vaccine DNL construct, including but not limited to radionuclides, immunomodulators, anti-angiogenic agents, cytokines,

chemokines, growth factors, hormones, drugs, prodrugs, enzymes, oligonucleotides, siRNAs, pro-apoptotic agents, photoactive therapeutic agents, cytotoxic agents, chemotherapeutic agents, toxins, other antibodies or antigen binding fragments thereof.

In a preferred embodiment, the therapeutic agent is a cytotoxic agent, such as a drug or a toxin. Also preferred, the drug is selected from the group consisting of nitrogen mustards, ethylenimine derivatives, alkyl sulfonates, nitrosoureas, gemcitabine, triazenes, folic acid analogs, anthracyclines, taxanes, COX-2 inhibitors, pyrimidine analogs, purine analogs, antibiotics, enzyme inhibitors, epipodophyllotoxins, platinum coordination complexes, vinca alkaloids, substituted ureas, methyl hydrazine derivatives, adrenocortical suppressants, hormone antagonists, endostatin, taxols, camptothecins, SN-38, doxorubicins and their analogs, antimetabolites, alkylating agents, antimitotics, anti-angiogenic agents, tyrosine kinase inhibitors, mTOR inhibitors, heat shock protein (HSP90) inhibitors, proteosome inhibitors, HDAC inhibitors, pro-apoptotic agents, methotrexate, CPT-11, and a combination thereof.

In another preferred embodiment, the therapeutic agent is a toxin selected from the group consisting of ricin, abrin, alpha toxin, saporin, ribonuclease (RNase), DNase I, Staphylococcal enterotoxin-A, pokeweed antiviral protein, gelonin, diphtheria toxin, *Pseudomonas* exotoxin, and *Pseudomonas* endotoxin and combinations thereof. Or an immunomodulator selected from the group consisting of a cytokine, a stem cell growth factor, a lymphotoxin, a hematopoietic factor, a colony stimulating factor (CSF), an interferon (IFN), a stem cell growth factor, erythropoietin, thrombopoietin and a combinations thereof.

In other preferred embodiments, the therapeutic agent is a radionuclide selected from the group consisting of ^{111}In, ^{177}Lu, ^{212}Bi, ^{213}Bi, ^{211}At, ^{62}Cu, ^{67}Cu, ^{90}Y, ^{125}I, ^{131}I, ^{32}P, ^{33}P, ^{47}Sc, ^{111}Ag, ^{67}Ga, ^{142}Pr, ^{153}Sm, ^{161}Tb, ^{166}Dy, ^{166}Ho, ^{186}Re, ^{188}Re, ^{189}Re, ^{212}Pb, ^{223}Ra, ^{225}Ac, ^{59}Fe, ^{75}Se, ^{77}As, ^{89}Sr, ^{99}Mo, ^{105}Rh, ^{109}Pd, ^{143}Pr, ^{149}Pm, ^{169}Er, ^{194}Ir, ^{198}Au, ^{199}Au, and ^{211}Pb, and combinations thereof. Also preferred are radionuclides that substantially decay with Auger-emitting particles. For example, Co-58, Ga-67, Br-80m, Tc-99m, Rh-103m, Pt-109, In-111, Sb-119, 1-125, Ho-161, Os-189m and Ir-192. Decay energies of useful beta-particle-emitting nuclides are preferably <1,000 keV, more preferably <100 keV, and most preferably <70 keV. Also preferred are radionuclides that substantially decay with generation of alpha-particles. Such radionuclides include, but are not limited to Dy-152, At-211, Bi-212, Ra-223, Rn-219, Po-215, Bi-211, Ac-225, Fr-221, At-217, Bi-213 and Fm-255. Decay energies of useful alpha-particle-emitting radionuclides are preferably 2,000-10,000 keV, more preferably 3,000-8,000 keV, and most preferably 4,000-7,000 keV. Additional potential radioisotopes of use include ^{11}C, ^{13}N, ^{15}O, ^{75}Br, ^{198}Au, ^{224}Ac, ^{126}I, ^{133}I, ^{77}Br, ^{113m}In, ^{95}Ru, ^{97}Ru, ^{103}Ru, ^{105}Ru, ^{107}Hg, ^{203}Hg, $_{121m}$Te, ^{122m}Te, ^{125m}Te, ^{165}Tm, ^{167}Tm, ^{168}Tm, ^{197}Pt, ^{109}Pd, ^{105}Rh, ^{142}Pr, ^{143}Pr, ^{161}Tb, ^{166}Ho, ^{199}Au, ^{57}Co, ^{58}Co, ^{51}Cr, ^{59}Fe, ^{75}Se, ^{201}Tl, ^{225}Ac, ^{76}Br, ^{169}Yb, and the like. In other embodiments the therapeutic agent is a photoactive therapeutic agent selected from the group consisting of chromogens and dyes.

Alternatively, the therapeutic agent is an enzyme selected from the group consisting of malate dehydrogenase, staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate

dehydrogenase, glucoamylase and acetylcholinesterase. Such enzymes may be used, for example, in combination with prodrugs that are administered in relatively non-toxic form and converted at the target site by the enzyme into a cytotoxic agent. In other alternatives, a drug may be converted into less toxic form by endogenous enzymes in the subject but may be reconverted into a cytotoxic form by the therapeutic enzyme.

Although in preferred embodiments, the anti-cancer vaccine DNL complexes are of use for therapy of multiple myeloma, the skilled artisan will realize that a CD20/anti-CD74 construct may potentially be of use for other types of diseases, such as other forms of CD20$^+$ cancer like B-cell lymphoma, B-cell leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, follicular lymphoma, mantle cell lymphoma, small lymphocytic lymphoma, diffuse B-cell lymphoma, marginal zone lymphoma, Burkitt lymphoma, Hodgkin's lymphoma or non-Hodgkin's lymphoma. Where a tumor-associated xenoantigen other than CD20 is used, the skilled artisan will realize that any type of cancer with an associated TAA may be targeted using the claimed DNL complexes.

Still other embodiments relate to DNA sequences encoding fusion proteins, such as antibody-DDD or xenoantigen-DDD fusion proteins or antibody-AD or xenoantigen-AD fusion proteins, vectors and host cells containing the DNA sequences, and methods of making fusion proteins for the production of anti-cancer vaccine DNL constructs. Related embodiments include fusion proteins of use for making anti-cancer vaccine DNL constructs, antibody-DDD or xenoantigen-DDD fusion proteins or antibody-AD or xenoantigen-AD fusion proteins. In alternative embodiments, the subunit components of the DNL complex may be formed by chemical cross-linking of, for example, an antibody or antibody fragment and a DDD peptide, or a CD20 xenoantigen and an AD peptide. For particular embodiments, the fusion protein or chemically cross-linked conjugate may be attached to a reporter moiety such as a diagnostic agent. A variety of diagnostic agents are known in the art, such as radionuclides, contrast agents, fluorescent agents, chemiluminescent agents, bioluminescent agents, paramagnetic ions, enzymes and photoactive diagnostic agents.

Preferably, the diagnostic agent is a radionuclide with an energy between 20 and 4,000 keV or is a radionuclide selected from the group consisting of ^{110}In, ^{111}In, ^{177}Lu, ^{18}F, ^{52}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{86}Y, ^{90}Y, ^{89}Zr, ^{94m}Tc, ^{94}Tc, ^{99m}Tc, ^{120}I, ^{123}I, ^{124}I, ^{125}I, ^{131}I, $^{154-158}$Gd, ^{32}P, ^{11}C, ^{13}N, ^{15}O, ^{186}Re, ^{188}Re, ^{51}Mn, ^{52m}Mn, ^{55}Co, ^{72}As, ^{75}Br, ^{76}Br, ^{82m}Rb, ^{83}Sr, or other gamma-, beta-, or positron-emitters.

Also preferred, the diagnostic agent is a paramagnetic ion, such as chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) and erbium (III), or a radiopaque material, such as barium, diatrizoate, ethiodized oil, gallium citrate, iocarmic acid, iocetamic acid, iodamide, iodipamide, iodoxamic acid, iogulamide, iohexyl, iopamidol, iopanoic acid, ioprocemic acid, iosefamic acid, ioseric acid, iosulamide meglumine, iosemetic acid, iotasul, iotetric acid, iothalamic acid, iotroxic acid, ioxaglic acid, ioxotrizoic acid, ipodate, meglumine, metrizamide, metrizoate, propyliodone, and thallous chloride.

In still other embodiments, the diagnostic agent is a fluorescent labeling compound selected from the group consisting of fluorescein isothiocyanate, rhodamine, phyco-

erytherin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine, a chemiluminescent labeling compound selected from the group consisting of luminol, isoluminol, an aromatic acridinium ester, an imidazole, an acridinium salt and an oxalate ester, or a bioluminescent compound selected from the group consisting of luciferin, luciferase and aequorin.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, the terms "a", "an" and "the" may refer to either the singular or plural, unless the context otherwise makes clear that only the singular is meant.

As used herein, the term "about" means plus or minus ten percent (10%) of a value. For example, "about 100" would refer to any number between 90 and 110.

An antibody refers to a full-length (i.e., naturally occurring or formed by normal immunoglobulin gene fragment recombinatorial processes) immunoglobulin molecule (e.g., an IgG antibody) or an immunologically active, antigen-binding portion of an immunoglobulin molecule, like an antibody fragment.

An antibody fragment is a portion of an antibody such as $F(ab')_2$, $F(ab)_2$, Fab', Fab, Fv, scFv and the like. Regardless of structure, an antibody fragment binds with the same antigen that is recognized by the intact antibody. Therefore the term is used synonymously with "antigen-binding antibody fragment." The term "antibody fragment" also includes isolated fragments consisting of the variable regions, such as the "Fv" fragments consisting of the variable regions of the heavy and light chains and recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker ("scFv proteins"). As used herein, the term "antibody fragment" does not include portions of antibodies without antigen binding activity, such as Fc fragments or single amino acid residues. Other antibody fragments, for example single domain antibody fragments, are known in the art and may be used in the claimed constructs. (See, e.g., Muyldermans et al., TIBS 26:230-235, 2001; Yau et al., J Immunol Methods 281:161-75, 2003; Maass et al., J Immunol Methods 324: 13-25, 2007).

The term antibody fusion protein may refer to a recombinantly produced antigen-binding molecule in which one or more of the same or different single-chain antibody or antibody fragment segments with the same or different specificities are linked. Valency of the fusion protein indicates how many binding arms or sites the fusion protein has to a single antigen or epitope; i.e., monovalent, bivalent, trivalent or multivalent. The multivalency of the antibody fusion protein means that it can take advantage of multiple interactions in binding to an antigen, thus increasing the avidity of binding to the antigen. Specificity indicates how many antigens or epitopes an antibody fusion protein is able to bind; i.e., monospecific, bispecific, trispecific, multispecific. Using these definitions, a natural antibody, e.g., an IgG, is bivalent because it has two binding arms but is monospecific because it binds to one epitope. Monospecific, multivalent fusion proteins have more than one binding site for an epitope but only bind with one epitope. The fusion protein may comprise a single antibody component, a multivalent or multispecific combination of different antibody components or multiple copies of the same antibody component. The fusion protein may additionally comprise an

antibody or an antibody fragment and a therapeutic agent. Examples of therapeutic agents suitable for such fusion proteins include immunomodulators and toxins. One preferred toxin comprises a ribonuclease (RNase), preferably a recombinant RNase. However, the term is not limiting and a variety of protein or peptide effectors may be incorporated into a fusion protein. In another non-limiting example, a fusion protein may comprise an AD or DDD sequence for producing a DNL construct as discussed below.

A chimeric antibody is a recombinant protein that contains the variable domains including the complementarity determining regions (CDRs) of an antibody derived from one species, preferably a rodent antibody, while the constant domains of the antibody molecule are derived from those of a human antibody. For veterinary applications, the constant domains of the chimeric antibody may be derived from that of other species, such as a cat or dog. A humanized antibody is a recombinant protein in which the CDRs from an antibody from one species; e.g., a rodent antibody, are transferred from the heavy and light variable chains of the rodent antibody into human heavy and light variable domains (e.g., framework region sequences). The constant domains of the antibody molecule are derived from those of a human antibody. In certain embodiments, a limited number of framework region amino acid residues from the parent (rodent) antibody may be substituted into the human antibody framework region sequences.

A human antibody is, e.g., an antibody obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain loci are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous murine heavy chain and light chain loci. The transgenic mice can synthesize human antibodies specific for particular antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described by Green et al., Nature Genet. 7:13 (1994), Lonberg et al., Nature 368:856 (1994), and Taylor et al., Int. Immun. 6:579 (1994). A fully human antibody also can be constructed by genetic or chromosomal transfection methods, as well as phage display technology, all of which are known in the art. See for example, McCafferty et al., Nature 348:552-553 (1990) for the production of human antibodies and fragments thereof in vitro, from immunoglobulin variable domain gene repertoires from unimmunized donors. In this technique, antibody variable domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. In this way, the phage mimics some of the properties of the B cell. Phage display can be performed in a variety of formats, for review, see e.g. Johnson and Chiswell, Current Opinion in Structural Biology 3:5564-571 (1993). Human antibodies may also be generated by in vitro activated B cells. See U.S. Pat. Nos. 5,567,610 and 5,229,275, the Examples section of which are incorporated herein by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Specific binding of hLL1 on human blood DC subsets, B cells, and monocytes. (A) The gating strategy for

the different APC subsets. (B) CD74 expression in APCs. (C) The binding efficiency of hLL1 on the cells. The numbers represent mean fluorescence intensity.

FIG. 2. CD74 expression in and binding efficiency of hLL1 with human monocyte-derived immature vs mature DCs. The human monocyte-derived DCs (day 5 after culture in the presence of hGM-CSF and hIL-4) were stained with FITC-labeled anti-CD74 antibody or AlexaFluor488-labeled hLL1, in combination with the staining with fluorescence-labeled mAbs against HLA-DR and CD83. The HLA-DR-positive cells are gated and analyzed. (A) CD74 expression in immature and LPS-matured DCs. (B) hLL1 binding with immature vs LPS-matured DCs. (C) Comparison of expression of CD83, HLA-DR, CD74 and hLL1 binding in immature and mature DCs.

FIG. 3. Side-by-side comparison of the cytotoxic effect of hLL1 on B cell malignant Daudi cells and normal DCs. (A) Comparison of the effect of hLL1 on Daudi and DCs. (B) Effect of hLL1 on cell viability of DCs in an extended doses. (C) The cytotoxic effect of hLL1 on Daudi cells. (D) The microscopic image shows no effect of hLL1 on DC viability.

FIG. 4. Moderate enhancement of DC constitutive maturation by hLL1. The HLA-DR positive cell populations were gated from day 5 DCs derived from human monocytes in the presence of hGM-CSF and hIL-4 (A) The expression of antigen-presenting molecule HLA-DR, costimulatory molecule CD54 and CD86 was measured by flow cytometry. (B) Expression levels of antigen-presenting molecule HLA-DR, costimulatory molecule CD54 and CD86.

FIG. 5. No significant influence of hLL1 on DC-mediated T cell proliferation. The hLL1-treated DCs were co-cultured with CFSE-labeled allogeneic PBMCs for 8 (A) or 11 days (B). The expanded T cells were stained with Percp-conjugated mAb against CD4. The cell proliferation of total T cells, CD4+ and CD4+ T cells were analyzed.

FIG. 6. Polarization of naïve CD4+ T cells by hLL1-treated DCs favoring the differentiation toward Th1 effector cells. Naïve CD4+ T cells isolated from human PBMCs using the depletion column with magnetic beads (MACS) were co-cultured with hLL1-treated allogeneic DCs. After different time points (day 11, 13, 18), the cells were harvested, stimulated with PMA and ionomycin, and analyzed with intracellular cytokine staining with fluorescence-labeled hIFN-gamma and hIL-4 antibodies. Th1/Th2/Th0 cells populations were gated and analyzed. The flow cytokine production in T cells stimulated by hLL1-treated DCs or by GAH-cross-linked hLL1-treated DCs was determined. (C) The data of Th1 responses in two donors, in the absence or presence of cross-linking by GAH, at different days after DC/T coculture, are shown. (D) The dose-effect curve for increasing Th1 populations by hLL1.

VACCINES FOR THERAPY OF MULTIPLE MYELOMA AND OTHER CANCERS

CD20 is normally expressed in cells of B cell lineage. It was recently reported that CD20 is expressed in a small population of MM cells isolated from MM cell lines or clinical specimens, which do not express the characteristic plasma cell surface antigen CD138 but have a highly clonogenic potential and are resistant to multiple clinical anti-myeloma drugs (Matsui et al., Blood 2004, 103:2332-6; Matsui et al., Cancer Res. 2008, 68:190-7). These CD20+ CD138-cells are capable of clonogenic growth in vitro and in a 3-D culture model (Kirshner et al., Blood 2008, 112: 2935-45), and of differentiation into MM cells in vitro and in the engrafted NOD/SCID mouse model during both

primary and secondary transplantation. It has thus been suggested that these $CD138^{neg}CD20^+$ cells represent the putative multiple myeloma cancer stem cells.

Immunization with Xenoantigen as a Means for Breaking Immune Tolerance for Cancer Immunotherapy.

Many tumor-associated Ags (TAAs) represent tissue differentiation Ags which are not inherently immunogenic. T cells that recognize these TAAs/self-Ags with high avidity are either clonally deleted in the thymus or anergized in the periphery. However, immunization with xenoantigen has been shown to be capable of overcoming the immune tolerance against the homologous self-Ag. In a phase I clinical trial, eleven of 21 prostate cancer patients immunized with dendritic cells pulsed with recombinant mouse PAP developed type I T-cell proliferative responses to the homologous self-Ag, and 6 patients had clinical stabilization of their previously progressing prostate cancer (Fong et al., J Immunol. 2001, 167(12):7150-6). These results demonstrate that xenoantigen immunization can break tolerance to a self-Ag in humans, resulting in a clinically significant antitumor effect.

CD20 as a Target for Immunotherapy and Vaccination Against MM.

As stated above, CD20 is a hallmark of MM cancer stem cells. As a self-antigen which is expressed on normal B cells at most stages of differentiation, it is theoretically difficult to be targeted by vaccine strategies due to immune tolerance. However, successful vaccination has been achieved by a xenogeneic DNA vaccine against CD20 in a tumor challenge model of B-cell lymphoma. Although autoimmunity against B cells could be induced by a vaccine targeting CD20, it should not cause a large problem because the B cell pool is not a vital and critical tissue and can be replenished from its lineage progenitor. Based on these considerations, a therapeutic vaccine targeting CD20 would be effective in selective eradication of MM cancer stem cells.

Monoclonal Anti-CD20 Antibody as a Potential Modality for Eradication of MM Stem Cells.

The discovery of CD20+ MM progenitor cells has prompted several small clinical trials to test the efficacy of rituximab, an anti-CD20 monoclonal antibody, in MM patients. As reviewed by Kapoor et al. (Br J Haematol. 2008, 141:135-48), anti-CD20 therapy with rituximab elicits a partial response in approximately 10% of CD20+ patients with multiple myeloma. In addition, there is preliminary evidence of disease stabilization in 50-57% of CD20+ patients for a period of 10-27 months (Kapoor et al., (Br J Haematol. 2008, 141:135-48). Furthermore, a case report by Bergua et al. (Leukemia. 2008, 22:1082-3) where rituximab was used in combination with chemotherapy demonstrated no minimal residual disease found after treatment, either in immunophenotype, bone marrow aspiration or biopsy, and the CD20+ plasma cells disappeared. These results justify large scale clinical trials to establish the role of this strategy in the treatment of myeloma. The vaccine approach, due to its induction of CTL response, would be expected to supplement the monoclonal antibody therapy against CD20 MM stem cells.

In Vivo Targeting of Antigens to Dendritic Cells and Other Antigen-Presenting Cells as an Efficient Strategy for Vaccination and Breaking Immune Tolerance.

As the professional antigen-presenting cells, dendritic cells (DCs) play a pivotal role in orchestrating innate and adaptive immunity, and have been harnessed to create effective vaccines (Vulink et al., Adv Cancer Res. 2008, 99:363-407; O'Neill et al., Mol Biotechnol. 2007, 36:131-41). In vivo targeting of antigens to DCs represents a promising

approach for DC-based vaccination, as it can bypass the laborious and expensive ex vivo antigen loading and culturing, and facilitate large-scale application of DC-based immunotherapy (Tacken et al., Nat Rev Immunol. 2007, 7:790-802). More significantly, in vivo DC targeting vaccination is more efficient in eliciting anti-tumor immune response, and more effective in controlling tumor growth in animal models (Kretz-Rommel et al., J Immunother 2007, 30:715-726). In addition to DCs, B cells are another type of potent antigen-presenting cells capable of priming Th1/Th2 cells (Morris et al, J Immunol. 1994, 152:3777-3785; Constant, J Immunol. 1999, 162:5695-5703) and activating CD8 T cells via cross-presentation (Heit et al., J Immunol. 2004, 172:1501-1507; Yan et al., Int Immunol. 2005, 17:869-773). It was recently reported that in vivo targeting of antigens to B cells breaks immune tolerance of MUC1 (Ding et al., Blood 2008, 112:2817-25).

CD74 as a Potential Receptor for Targeting Vaccination.

Some receptors expressed on DCs have been used as the targets for in vivo antigen targeting, such as the mannose receptor (He et al., J. Immunol 2007, 178, 6259-6267; Ramakrishna et al., J. Immunol. 2004, 172, 2845-2852) CD205 (Bonifaz et al., J Exp Med. 2004, 199:815-24), DC-SIGN (Tacken et al., Blood 2005, 106:1278-85), and LOX1 (Deineste et al., Immunity 2002, 17, 353-362), etc. CD74 is a type II integral membrane protein essential for proper MHC II folding and targeting of MHC II-CD74 complex to the endosomes (Stein et al., Clin Cancer Res. 2007, 13:5556s-5563s; Matza et al., Trends Immunol. 2003, 24(5):264-8). CD74 expression is not restricted to DCs, but is found in almost all antigen-presenting cells (Freudenthal et al., Proc Natl Acad Sci USA. 1990, 87:7698-702; Clark et al., J Immunol. 1992, 148(11):3327-35). The wide expression of CD74 in APCs may offer some advantages over sole expression in myeloid DCs, as targeting of antigens to other APCs like B cells has been reported to break immune tolerance (Ding et al., Blood 2008, 112:2817-25), and targeting to plasmacytoid DCs cross-presents antigens to naïve CD8 T cells. More importantly, CD74 is also expressed in follicular DCs (Clark et al., J Immunol. 1992, 148(11):3327-35), a DC subset critical for antigen presentation to B cells (Tew et al., Immunol Rev. 1997, 156:39-52). This expression profile makes CD74 an excellent candidate for in vivo targeting vaccination.

Humanized Anti-CD74 Monoclonal Antibody hLL1 as a Novel Targeting Tool with Dock-and-Lock Technology Platform.

The DNL technology, discussed in more detail below, provides a means to link virtually any selected effector moieties into a covalent or noncovalent complex (Goldenberg et al., J Nucl Med. 2008, 49:158-63; Rossi et al., Proc Natl Acad Sci USA. 2006, 103(18):6841-6). The DNL method has generated several trivalent, bispecific, binding proteins containing Fab fragments reacting with carcinoembryonic antigen (CEA), and has been successfully used in improved cancer imaging and radioimmunotherapy through a pretargeting strategy (Goldenberg et al., J Nucl Med. 2008, 49:158-63).

hLL1 is a humanized monoclonal antibody against human CD74 (Leung et al., Mol Immunol. 1995, 32:1416-1427; Losman et al., Cancer 1997, 80:2660-2666; Stein et al., Blood 2004, 104:3705-11). This MAb, in the presence of cross-linking by a second antibody, exhibits cytotoxicity against B cell malignancies. The naked hLL1 is also capable of controlling tumor growth in a MM mouse model. However, our recent data demonstrate that hLL1, in the presence or absence of cross-linking, has no cytotoxicity against

human monocyte-derived DCs. But, our preliminary data shows hLL1 could efficiently bind different subsets of blood DCs and B cells. It also could moderately induce DC maturation and polarize naïve T cell differentiation toward Th1 effector cells, suggesting it has some adjuvant activity and may be a good candidate for use as a targeting tool. This makes it possible and feasible to construct a DNL-based tumor vaccine targeted to APCs through the DNL-carried hLL1 antibody.

Immunotherapy for Selective Elimination of Cancer Stem Cells.

Cancer stem cells are capable of self-renewal, possess the ability for unlimited proliferation, and are resistant to multiple therapeutic approaches. A pressing and interesting question is raised if cancer stem cells are sensitive to immunotherapy. In the case of leukemia, it was reported that CD8(+) minor histocompatibility antigen-specific cytotoxic T lymphocyte clones could eliminate human acute myeloid leukemia stem cells (Bonnet et al., Proc Natl Acad Sci U.S.A. 1999, 96:8639-8644). More recently, Rosinski et al. (Blood 2008, 111:4817-26) reported that DDX36-encoded H—Y epitope is expressed by leukemic stem cells and can be recognized by the DDX36-specific CTLs, which can prevent engraftment of human acute leukemia in NOD/ SCID mice (Rosinski et al. Blood 2008, 111:4817-26). Another report indicates that engraftment of mHA myeloid leukemia stem cells in NOD/SCIDγc^{null} mice was completely inhibited by in vitro preincubation with the mHA-specific CTL clone (Kawase et al., Blood 2007, 110:1055-63). These results highlight the prospects that immunotherapy would be a potentially effective approach for selective elimination of cancer stem cells including MM stem cells, which would be required for achieving long-term control or even cure of this malignancy.

Dock and Lock (DNL) Method

The DNL method exploits specific protein/protein interactions that occur between the regulatory (R) subunits of cAMP-dependent protein kinase (PKA) and the anchoring domain (AD) of A-kinase anchoring proteins (AKAPs) (Baillie et al., FEBS Letters. 2005; 579:3264. Wong and Scott, Nat. Rev. Mol. Cell Biol. 2004; 5:959). PKA, which plays a central role in one of the best studied signal transduction pathways triggered by the binding of the second messenger cAMP to the R subunits, was first isolated from rabbit skeletal muscle in 1968 (Walsh et al., J. Biol. Chem. 1968; 243:3763). The structure of the holoenzyme consists of two catalytic subunits held in an inactive form by the R subunits (Taylor, J. Biol. Chem. 1989; 264:8443). Isozymes of PKA are found with two types of R subunits (RI and RID, and each type has α and β isoforms (Scott, Pharmacol. Ther. 1991; 50:123). The R subunits have been isolated only as stable dimers and the dimerization domain has been shown to consist of the first 44 amino-terminal residues (Newlon et al., Nat. Struct. Biol. 1999; 6:222). Binding of cAMP to the R subunits leads to the release of active catalytic subunits for a broad spectrum of serine/threonine kinase activities, which are oriented toward selected substrates through the compartmentalization of PKA via its docking with AKAPs (Scott et al., J. Biol. Chem. 1990; 265; 21561)

Since the first AKAP, microtubule-associated protein-2, was characterized in 1984 (Lohmann et al., Proc. Natl. Acad. Sci USA. 1984; 81:6723), more than 50 AKAPs that localize to various sub-cellular sites, including plasma membrane, actin cytoskeleton, nucleus, mitochondria, and endoplasmic reticulum, have been identified with diverse structures in species ranging from yeast to humans (Wong and Scott, Nat. Rev. Mol. Cell Biol. 2004; 5:959). The AD of AKAPs for

PKA is an amphipathic helix of 14-18 residues (Carr et al., J. Biol. Chem. 1991; 266:14188). The amino acid sequences of the AD are quite varied among individual AKAPs, with the binding affinities reported for RII dimers ranging from 2 to 90 nM (Alto et al., Proc. Natl. Acad. Sci. USA. 2003; 100:4445). Interestingly, AKAPs will only bind to dimeric R subunits. For human RIIα, the AD binds to a hydrophobic surface formed by the 23 amino-terminal residues (Colledge and Scott, Trends Cell Biol. 1999; 6:216). Thus, the dimerization domain and AKAP binding domain of human RIIα are both located within the same N-terminal 44 amino acid sequence (Newlon et al., Nat. Struct. Biol. 1999; 6:222; Newlon et al., EMBO J. 2001; 20:1651), which is termed the DDD herein.

DDD of Human RIIα and AD of AKAPs as Linker Modules

We have developed a platform technology to utilize the DDD of human RIIα and the AD of a AKAPs as an excellent pair of linker modules for docking any two entities, referred to hereafter as A and B, into a noncovalent complex, which could be further locked into a stably tethered structure through the introduction of cysteine residues into both the DDD and AD at strategic positions to facilitate the formation of disulfide bonds. The general methodology of the "dock-and-lock" approach is as follows. Entity A is constructed by linking a DDD sequence to a precursor of A, resulting in a first component hereafter referred to as a. Because the DDD sequence would effect the spontaneous formation of a dimer, A would thus be composed of a_2. Entity B is constructed by linking an AD sequence to a precursor of B, resulting in a second component hereafter referred to as b. The dimeric motif of DDD contained in a_2 will create a docking site for binding to the AD sequence contained in b, thus facilitating a ready association of a_2 and b to form a binary, trimeric complex composed of a_2b. This binding event is made irreversible with a subsequent reaction to covalently secure the two entities via disulfide bridges, which occurs very efficiently based on the principle of effective local concentration because the initial binding interactions should bring the reactive thiol groups placed onto both the DDD and AD into proximity (Chmura et al., Proc. Natl. Acad. Sci. USA. 2001; 98:8480) to ligate site-specifically.

In preferred embodiments, the anti-cancer vaccine DNL constructs are based on a variation of the a_2b structure, in which each heavy chain of an anti-CD74 antibody or $F(ab')_2$ or $F(ab)_2$ antibody fragment, such as an hLL1 antibody or fragment, is attached at its C-terminal end to one copy of an AD moiety. Since there are two heavy chains per antibody or fragment, there are two AD moieties per antibody or fragment. A CD20 xenoantigen is attached to a complementary DDD moiety. After dimerization of DDD moieties, each DDD dimer binds to one of the AD moieties attached to the IgG antibody or $F(ab')_2$ or $F(ab)_2$ fragment, resulting in a stoichiometry of four CD20 xenoantigens per IgG or $F(ab')_2$ or $F(ab)_2$ unit. However, the skilled artisan will realize that alternative complexes may be utilized, such as attachment of the CD20 to the AD sequence and attachment of the anti-CD74 MAb or fragment to the DDD moiety, resulting in a different stoichiometry of effector moieties. For example, by attaching a DDD sequence to the C-terminal end of each heavy chain of an IgG antibody or $F(ab')_2$ fragment, and attaching an AD sequence to the CD20 xenoantigen, a DNL complex may be constructed that comprises one CD20 molecule and one anti-CD74 antibody or fragment.

By attaching the DDD and AD away from the functional groups of the two precursors, such site-specific ligations are expected to preserve the original activities of the two

precursors. This approach is modular in nature and potentially can be applied to link, site-specifically and covalently, a wide range of substances.

In preferred embodiments, as illustrated in the Examples below, the effector moiety is a protein or peptide, which can be linked to a DDD or AD unit to form a fusion protein or peptide. A variety of methods are known for making fusion proteins, including nucleic acid synthesis, hybridization and/or amplification to produce a synthetic double-stranded nucleic acid encoding a fusion protein of interest. Such double-stranded nucleic acids may be inserted into expression vectors for fusion protein production by standard molecular biology techniques (see, e.g. Sambrook et al., *Molecular Cloning, A laboratory manual, 2nd* Ed, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 1989). In such preferred embodiments, the AD and/or DDD moiety may be attached to either the N-terminal or C-terminal end of an effector protein or peptide. However, the skilled artisan will realize that the site of attachment of an AD or DDD moiety to an effector moiety may vary, depending on the chemical nature of the effector moiety and the part(s) of the effector moiety involved in its physiological activity. For example, although an AD or DDD moiety may be attached to either the N- or C-terminal end of an antibody or antibody fragment while retaining antigen-binding activity, attachment to the C-terminal end positions the AD or DDD moiety farther from the antigen-binding site and appears to result in a stronger binding interaction (e.g., Chang et al., Clin Cancer Res 2007, 13:5586s-91s). Site-specific attachment of a variety of effector moieties may be also performed using techniques known in the art, such as the use of bivalent cross-linking reagents and/or other chemical conjugation techniques.

Antibodies and Antibody Fragments

In various embodiments, antibodies or antigen-binding fragments of antibodies may be incorporated into the anti-cancer vaccine DNL complex. Antigen-binding antibody fragments are well known in the art, such as $F(ab')_2$, $F(ab)_2$, Fab', Fab, Fv, scFv and the like, and any such known fragment may be used. As used herein, an antigen-binding antibody fragment refers to any fragment of an antibody that binds with the same antigen that is recognized by the intact or parent antibody. Techniques for preparing AD and/or DDD conjugates of virtually any antibody or fragment of interest are known (e.g., U.S. Pat. No. 7,527,787).

An antibody or fragment thereof may be used which is not conjugated to a therapeutic agent—referred to as a "naked" antibody or fragment thereof. In alternative embodiments, antibodies or fragments may be conjugated to one or more therapeutic and/or diagnostic agents. A wide variety of such therapeutic and diagnostic agents are known in the art, as discussed in more detail below, and any such known therapeutic or diagnostic agent may be used.

Techniques for preparing monoclonal antibodies against virtually any target antigen, such as CD74, are well known in the art. See, for example, Kohler and Milstein, *Nature* 256:495 (1975), and Coligan et al. (eds.), CURRENT PROTOCOLS IN IMMUNOLOGY, VOL. 1, pages 2.5.1-2.6.7 (John Wiley & Sons 1991). Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, removing the spleen to obtain B-lymphocytes, fusing the B-lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones which produce antibodies to the antigen, culturing the clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures.

MAbs can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. See, for example, Coligan at pages 2.7.1-2.7.12 and pages 2.9.1-2.9.3. Also, see Baines et al., "Purification of Immunoglobulin G (IgG)," in METHODS IN MOLECULAR BIOLOGY, VOL. 10, pages 79-104 (The Humana Press, Inc. 1992).

After the initial raising of antibodies to the immunogen, the antibodies can be sequenced and subsequently prepared by recombinant techniques. Humanization and chimerization of murine antibodies and antibody fragments are well known to those skilled in the art. The use of antibody components derived from humanized, chimeric or human antibodies obviates potential problems associated with the immunogenicity of murine constant regions.

Chimeric Antibodies

A chimeric antibody is a recombinant protein in which the variable regions of a human antibody have been replaced by the variable regions of, for example, a mouse antibody, including the complementarity-determining regions (CDRs) of the mouse antibody. Chimeric antibodies exhibit decreased immunogenicity and increased stability when administered to a subject. General techniques for cloning murine immunoglobulin variable domains are disclosed, for example, in Orlandi et al., *Proc. Nat'l Acad. Sci. USA* 86:3833 (1989). Techniques for constructing chimeric antibodies are well known to those of skill in the art. As an example, Leung et al., *Hybridoma* 13:469 (1994), produced an LL2 chimera by combining DNA sequences encoding the V_κ and V_H domains of murine LL2, an anti-CD22 monoclonal antibody, with respective human κ and IgG_1 constant region domains.

Humanized Antibodies

Techniques for producing humanized MAbs are well known in the art (see, e.g., Jones et al., *Nature* 321:522 (1986), Riechmann et al., *Nature* 332:323 (1988), Verhoeyen et al., *Science* 239:1534 (1988), Carter et al., *Proc. Nat'l Acad. Sci. USA* 89:4285 (1992), Sandhu, *Crit. Rev. Biotech.* 12:437 (1992), and Singer et al., *J. Immun.* 150:2844 (1993)). A chimeric or murine monoclonal antibody may be humanized by transferring the mouse CDRs from the heavy and light variable chains of the mouse immunoglobulin into the corresponding variable domains of a human antibody. The mouse framework regions (FR) in the chimeric monoclonal antibody are also replaced with human FR sequences. As simply transferring mouse CDRs into human FRs often results in a reduction or even loss of antibody affinity, additional modification might be required in order to restore the original affinity of the murine antibody. This can be accomplished by the replacement of one or more human residues in the FR regions with their murine counterparts to obtain an antibody that possesses good binding affinity to its epitope. See, for example, Tempest et al., *Biotechnology* 9:266 (1991) and Verhoeyen et al., *Science* 239:1534 (1988). Generally, those human FR amino acid residues that differ from their murine counterparts and are located close to or touching one or more CDR amino acid residues would be candidates for substitution.

A humanized LL1 (hLL1) anti-CD74 antibody is disclosed in U.S. Pat. No. 7,312,318, incorporated herein by reference from Col. 35, line 1 through Col. 42, line 27 and FIG. 1 through FIG. 4.

Human Antibodies

Methods for producing fully human antibodies using either combinatorial approaches or transgenic animals trans-

formed with human immunoglobulin loci are known in the art (e.g., Mancini et al., 2004, New Microbiol. 27:315-28; Conrad and Scheller, 2005, *Comb. Chem. High Throughput Screen.* 8:117-26; Brekke and Loset, 2003, *Curr. Opin. Phamacol.* 3:544-50). A fully human antibody also can be constructed by genetic or chromosomal transfection methods, as well as phage display technology, all of which are known in the art. See for example, McCafferty et al., *Nature* 348:552-553 (1990). Such fully human antibodies are expected to exhibit even fewer side effects than chimeric or humanized antibodies and to function in vivo as essentially endogenous human antibodies. In certain embodiments, the claimed methods and procedures may utilize human antibodies produced by such techniques.

In one alternative, the phage display technique may be used to generate human antibodies (e.g., Dantas-Barbosa et al., 2005, *Genet. Mol. Res.* 4:126-40). Human antibodies may be generated from normal humans or from humans that exhibit a particular disease state, such as cancer (Dantas-Barbosa et al., 2005). The advantage to constructing human antibodies from a diseased individual is that the circulating antibody repertoire may be biased towards antibodies against disease-associated antigens.

In one non-limiting example of this methodology, Dantas-Barbosa et al. (2005) constructed a phage display library of human Fab antibody fragments from osteosarcoma patients. Generally, total RNA was obtained from circulating blood lymphocytes (Id.). Recombinant Fab were cloned from the μ, γ and κ chain antibody repertoires and inserted into a phage display library (Id.). RNAs were converted to cDNAs and used to make Fab cDNA libraries using specific primers against the heavy and light chain immunoglobulin sequences (Marks et al., 1991, *J. Mol. Biol.* 222:581-97). Library construction was performed according to Andris-Widhopf et al. (2000, In: *Phage Display Laboratory Manual*, Barbas et al. (eds), 1st edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. pp. 9.1 to 9.22). The final Fab fragments were digested with restriction endonucleases and inserted into the bacteriophage genome to make the phage display library. Such libraries may be screened by standard phage display methods, as known in the art (see, e.g., Pasqualini and Ruoslahti, 1996, Nature 380:364-366; Pasqualini, 1999, The Quart. J. Nucl. Med. 43:159-162).

Phage display can be performed in a variety of formats, for their review, see e.g. Johnson and Chiswell, *Current Opinion in Structural Biology* 3:5564-571 (1993). Human antibodies may also be generated by in vitro activated B-cells. See U.S. Pat. Nos. 5,567,610 and 5,229,275, incorporated herein by reference in their entirety. The skilled artisan will realize that these techniques are exemplary and any known method for making and screening human antibodies or antibody fragments may be utilized.

In another alternative, transgenic animals that have been genetically engineered to produce human antibodies may be used to generate antibodies against essentially any immunogenic target, using standard immunization protocols. Methods for obtaining human antibodies from transgenic mice are disclosed by Green et al., *Nature Genet.* 7:13 (1994), Lonberg et al., *Nature* 368:856 (1994), and Taylor et al., *Int. Immun.* 6:579 (1994). A non-limiting example of such a system is the XenoMouse® (e.g., Green et al., 1999, *J. Immunol. Methods* 231:11-23) from Abgenix (Fremont, Calif.). In the XenoMouse® and similar animals, the mouse antibody genes have been inactivated and replaced by functional human antibody genes, while the remainder of the mouse immune system remains intact.

The XenoMouse® was transformed with germline-configured YACs (yeast artificial chromosomes) that contained portions of the human IgH and Igkappa loci, including the majority of the variable region sequences, along with accessory genes and regulatory sequences. The human variable region repertoire may be used to generate antibody producing B-cells, which may be processed into hybridomas by known techniques. A XenoMouse® immunized with a target antigen will produce human antibodies by the normal immune response, which may be harvested and/or produced by standard techniques discussed above. A variety of strains of XenoMouse® are available, each of which is capable of producing a different class of antibody. Transgenically produced human antibodies have been shown to have therapeutic potential, while retaining the pharmacokinetic properties of normal human antibodies (Green et al., 1999). The skilled artisan will realize that the claimed compositions and methods are not limited to use of the XenoMouse® system but may utilize any transgenic animal that has been genetically engineered to produce human antibodies.

Antibody Fragments

Antibody fragments which recognize specific epitopes can be generated by known techniques. Antibody fragments are antigen binding portions of an antibody, such as F(ab')$_2$, Fab', F(ab)$_2$, Fab, Fv, sFv and the like. F(ab')$_2$ fragments can be produced by pepsin digestion of the antibody molecule and Fab' fragments can be generated by reducing disulfide bridges of the F(ab')$_2$ fragments. Alternatively, Fab' expression libraries can be constructed (Huse et al., 1989, *Science*, 246:1274-1281) to allow rapid and easy identification of monoclonal Fab' fragments with the desired specificity. F(ab)$_2$ fragments may be generated by papain digestion of an antibody and Fab fragments obtained by disulfide reduction.

A single chain Fv molecule (scFv) comprises a VL domain and a VH domain. The VL and VH domains associate to form a target binding site. These two domains are further covalently linked by a peptide linker (L). Methods for making scFv molecules and designing suitable peptide linkers are described in U.S. Pat. No. 4,704,692, U.S. Pat. No. 4,946,778, R. Raag and M. Whitlow, "*Single Chain Fvs.*" FASEB Vol 9:73-80 (1995) and R. E. Bird and B. W. Walker, "*Single Chain Antibody Variable Regions,*" TIBTECH, Vol 9:132-137 (1991).

Techniques for producing single domain antibodies are also known in the art, as disclosed for example in Cossins et al. (2006, Prot Express Purif 51:253-259). Single domain antibodies (VHH) may be obtained, for example, from camels, alpacas or llamas by standard immunization techniques. (See, e.g., Muyldermans et al., TIBS 26:230-235, 2001; Yau et al., J Immunol Methods 281:161-75, 2003; Maass et al., J Immunol Methods 324:13-25, 2007). The VHH may have potent antigen-binding capacity and can interact with novel epitopes that are inaccessible to conventional VH-VL pairs. (Muyldermans et al., 2001). Alpaca serum IgG contains about 50% camelid heavy chain only IgG antibodies (HCAbs) (Maass et al., 2007). Alpacas may be immunized with known antigens, such as TNF-α, and VHHs can be isolated that bind to and neutralize the target antigen (Maass et al., 2007). PCR primers that amplify virtually all alpaca VHH coding sequences have been identified and may be used to construct alpaca VHH phage display libraries, which can be used for antibody fragment isolation by standard biopanning techniques well known in the art (Maass et al., 2007).

An antibody fragment can be prepared by proteolytic hydrolysis of the full length antibody or by expression in *E. coli* or another host of the DNA coding for the fragment. An antibody fragment can be obtained by pepsin or papain digestion of full length antibodies by conventional methods. These methods are described, for example, by Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647 and references contained therein. Also, see Nisonoff et al., *Arch Biochem. Biophys.* 89:230 (1960); Porter, *Biochem. J.* 73:119 (1959), Edelman et al., in METHODS IN ENZYMOLOGY VOL. 1, page 422 (Academic Press 1967), and Coligan at pages 2.8.1-2.8.10 and 2.10.-2.10.4.

Known Antibodies

In certain embodiments antibodies against other antigenic targets besides CD74 may be incorporated into the anti-cancer vaccine DNL complex. A wide variety of antibodies against tumor-associated antigens are known and may be obtained from commercial sources. For example, a number of antibody secreting hybridoma lines are available from the American Type Culture Collection (ATCC, Manassas, Va.). See, e.g., U.S. Pat. Nos. 7,312,318; 7,282,567; 7,151,164; 7,074,403; 7,060,802; 7,056,509; 7,049,060; 7,045,132; 7,041,803; 7,041,802; 7,041,293; 7,038,018; 7,037,498; 7,012,133; 7,001,598; 6,998,468; 6,994,976; 6,994,852; 6,989,241; 6,974,863; 6,965,018; 6,964,854; 6,962,981; 6,962,813; 6,956,107; 6,951,924; 6,949,244; 6,946,129; 6,943,020; 6,939,547; 6,921,645; 6,921,645; 6,921,533; 6,919,433; 6,919,078; 6,916,475; 6,905,681; 6,899,879; 6,893,625; 6,887,468; 6,887,466; 6,884,594; 6,881,405; 6,878,812; 6,875,580; 6,872,568; 6,867,006; 6,864,062; 6,861,511; 6,861,227; 6,861,226; 6,838,282; 6,835,549; 6,835,370; 6,824,780; 6,824,778; 6,812,206; 6,793,924; 6,783,758; 6,770,450; 6,767,711; 6,764,688; 6,764,681; 6,764,679; 6,743,898; 6,733,981; 6,730,307; 6,720,155; 6,716,966; 6,709,653; 6,693,176; 6,692,908; 6,689,607; 6,689,362; 6,689,355; 6,682,737; 6,682,736; 6,682,734; 6,673,344; 6,653,104; 6,652,852; 6,635,482; 6,630,144; 6,610,833; 6,610,294; 6,605,441; 6,605,279; 6,596,852; 6,592,868; 6,576,745; 6,572,856; 6,566,076; 6,562,618; 6,545,130; 6,544,749; 6,534,058; 6,528,625; 6,528,269; 6,521,227; 6,518,404; 6,511,665; 6,491,915; 6,488,930; 6,482,598; 6,482,408; 6,479,247; 6,468,531; 6,468,529; 6,465,173; 6,461,823; 6,458,356; 6,455,044; 6,455,040; 6,451,310; 6,444,206; 6,441,143; 6,432,404; 6,432,402; 6,419,928; 6,413,726; 6,406,694; 6,403,770; 6,403,091; 6,395,276; 6,395,274; 6,387,350; 6,383,759; 6,383,484; 6,376,654; 6,372,215; 6,359,126; 6,355,481; 6,355,444; 6,355,245; 6,355,244; 6,346,246; 6,344,198; 6,340,571; 6,340,459; 6,331,175; 6,306,393; 6,254,868; 6,187,287; 6,183,744; 6,129,914; 6,120,767; 6,096,289; 6,077,499; 5,922,302; 5,874,540; 5,814,440; 5,798,229; 5,789,554; 5,776,456; 5,736,119; 5,716,595; 5,677,136; 5,587,459; 5,443,953, 5,525,338. These are exemplary only and a wide variety of other antibodies and their hybridomas are known in the art. The skilled artisan will realize that antibody sequences or antibody-secreting hybridomas against almost any tumor-associated antigen may be obtained by a simple search of the ATCC, NCBI and/or USPTO databases for antibodies against a selected disease-associated target of interest. The antigen binding domains of the cloned antibodies may be amplified, excised, ligated into an expression vector, transfected into an adapted host cell and used for protein production, using standard techniques well known in the art.

Amino Acid Substitutions

In certain embodiments, the disclosed methods and compositions may involve production and use of proteins or peptides with one or more substituted amino acid residues. For example, as discussed in the working Examples below the sequences of the AD and/or DDD moieties may be varied

to improve DNL complex formation and/or in vivo stability of the DNL complexes. In other embodiments, the structural, physical and/or therapeutic characteristics of native, chimeric, humanized or human antibodies may be optimized by replacing one or more amino acid residues. For example, it is well known in the art that the functional characteristics of humanized antibodies may be improved by substituting a limited number of human framework region (FR) amino acids with the corresponding FR amino acids of the parent murine antibody. This is particularly true when the framework region amino acid residues are in close proximity to the CDR residues.

In other cases, the therapeutic properties of an antibody, such as binding affinity for the target antigen, the dissociation- or off-rate of the antibody from its target antigen, or even the effectiveness of induction of CDC (complement-dependent cytotoxicity) or ADCC (antibody dependent cellular cytotoxicity) by the antibody, may be optimized by a limited number of amino acid substitutions.

The skilled artisan will be aware that, in general, amino acid substitutions typically involve the replacement of an amino acid with another amino acid of relatively similar properties (i.e., conservative amino acid substitutions). The properties of the various amino acids and effect of amino acid substitution on protein structure and function have been the subject of extensive study and knowledge in the art.

For example, the hydropathic index of amino acids may be considered (Kyte & Doolittle, 1982, J. Mol. Biol., 157: 105-132). The relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte & Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (−0.4); threonine (−0.7); serine (−0.8); tryptophan (−0.9); tyrosine (−1.3); proline (−1.6); histidine (−3.2); glutamate (−3.5); glutamine (−3.5); aspartate (−3.5); asparagine (−3.5); lysine (−3.9); and arginine (−4.5). In making conservative substitutions, the use of amino acids whose hydropathic indices are within ±2 is preferred, within ±1 are more preferred, and within ±0.5 are even more preferred.

Amino acid substitution may also take into account the hydrophilicity of the amino acid residue (e.g., U.S. Pat. No. 4,554,101). Hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0); glutamate (+3.0); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (−0.4); proline (−0.5+−1); alanine (−0.5); histidine (−0.5); cysteine (−1.0); methionine (−1.3); valine (−1.5); leucine (−1.8); isoleucine (−1.8); tyrosine (−2.3); phenylalanine (−2.5); tryptophan (−3.4). Replacement of amino acids with others of similar hydrophilicity is preferred.

Other considerations include the size of the amino acid side chain. For example, it would generally not be preferred to replace an amino acid with a compact side chain, such as glycine or serine, with an amino acid with a bulky side chain, e.g., tryptophan or tyrosine. The effect of various amino acid residues on protein secondary structure is also a consideration. Through empirical study, the effect of different amino acid residues on the tendency of protein domains to adopt an alpha-helical, beta-sheet or reverse turn secondary structure has been determined and is known in the art

(see, e.g., Chou & Fasman, 1974, Biochemistry, 13:222-245; 1978, Ann. Rev. Biochem., 47:251-276; 1979, Biophys. J., 26:367-384).

Based on such considerations and extensive empirical study, tables of conservative amino acid substitutions have been constructed and are known in the art. For example arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine. Alternatively Ala (A) leu, ile, val; Arg (R) gln, asn, lys; Asn (N) his, asp, lys, arg, gln; Asp (D) asn, glu; Cys (C) ala, ser; Gln (Q) glu, asn; Glu (E) gln, asp; Gly (G) ala; H is (H) asn, gln, lys, arg; He (I) val, met, ala, phe, leu; Leu (L) val, met, ala, phe, ile; Lys (K) gln, asn, arg; Met (M) phe, ile, leu; Phe (F) leu, val, ile, ala, tyr; Pro (P) ala; Ser (S), thr; Thr (T) ser; Trp (W) phe, tyr; Tyr (Y) trp, phe, thr, ser; Val (V) ile, leu, met, phe, ala.

Other considerations for amino acid substitutions include whether or not the residue is located in the interior of a protein or is solvent exposed. For interior residues, conservative substitutions would include Asp and Asn; Ser and Thr; Ser and Ala; Thr and Ala; Ala and Gly; Ile and Val; Val and Leu; Leu and Ile; Leu and Met; Phe and Tyr; Tyr and Trp. (See, e.g., PROWL website at rockefeller.edu) For solvent exposed residues, conservative substitutions would include Asp and Asn; Asp and Glu; Glu and Gln; Glu and Ala; Gly and Asn; Ala and Pro; Ala and Gly; Ala and Ser; Ala and Lys; Ser and Thr; Lys and Arg; Val and Leu; Leu and Ile; Ile and Val; Phe and Tyr. (See, e.g., PROWL website at rockefeller.edu) Various matrices have been constructed to assist in selection of amino acid substitutions, such as the PAM250 scoring matrix, Dayhoff matrix, Grantham matrix, McLachlan matrix, Doolittle matrix, Henikoff matrix, Miyata matrix, Fitch matrix, Jones matrix, Rao matrix, Levin matrix and Risler matrix (See, e.g., PROWL website at rockefeller.edu)

In determining amino acid substitutions, one may also consider the existence of intermolecular or intramolecular bonds, such as formation of ionic bonds (salt bridges) between positively charged residues (e.g., His, Arg, Lys) and negatively charged residues (e.g., Asp, Glu) or disulfide bonds between nearby cysteine residues.

Methods of substituting any amino acid for any other amino acid in an encoded protein sequence are well known and a matter of routine experimentation for the skilled artisan, for example by the technique of site-directed mutagenesis or by synthesis and assembly of oligonucleotides encoding an amino acid substitution and splicing into an expression vector construct. (E.g., Sambrook et al., *Molecular Cloning, A laboratory manual*, 2nd Ed, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 1989.)

Therapeutic Agents

In certain embodiments, therapeutic agents such as cytotoxic agents, anti-angiogenic agents, pro-apoptotic agents, antibiotics, hormones, hormone antagonists, chemokines, drugs, prodrugs, toxins, enzymes or other agents may be used as adjunct therapies to the anti-cancer vaccine DNL complexes described herein. Drugs of use may possess a pharmaceutical property selected from the group consisting of antimitotic, antikinase, alkylating, antimetabolite, antibiotic, alkaloid, anti-angiogenic, pro-apoptotic agents and combinations thereof.

Exemplary drugs of use may include 5-fluorouracil, aplidin, azaribine, anastrozole, anthracyclines, bendamustine, bleomycin, bortezomib, bryostatin-1, busulfan, calicheamycin, camptothecin, carboplatin, 10-hydroxycamptothecin, carmustine, celebrex, chlorambucil, cisplatin (CDDP), Cox-2 inhibitors, irinotecan (CPT-11), SN-38, carboplatin,

cladribine, camptothecans, cyclophosphamide, cytarabine, dacarbazine, docetaxel, dactinomycin, daunorubicin, doxorubicin, 2-pyrrolinodoxorubicine (2P-DOX), cyano-morpholino doxorubicin, doxorubicin glucuronide, epirubicin glucuronide, estramustine, epidophyllotoxin, estrogen receptor binding agents, etoposide (VP16), etoposide glucuronide, etoposide phosphate, floxuridine (FUdR), 3',5'-O-dioleoyl-FudR (FUdR-dO), fludarabine, flutamide, farnesyl-protein transferase inhibitors, gemcitabine, hydroxyurea, idarubicin, ifosfamide, L-asparaginase, lenolidamide, leucovorin, lomustine, mechlorethamine, melphalan, mercaptopurine, 6-mercaptopurine, methotrexate, mitoxantrone, mithramycin, mitomycin, mitotane, navelbine, nitrosurea, plicomycin, procarbazine, paclitaxel, pentostatin, PSI-341, raloxifene, semustine, streptozocin, tamoxifen, taxol, temazolomide (an aqueous form of DTIC), transplatinum, thalidomide, thioguanine, thiotepa, teniposide, topotecan, uracil mustard, vinorelbine, vinblastine, vincristine and vinca alkaloids.

Toxins of use may include ricin, abrin, alpha toxin, saporin, ribonuclease (RNase), e.g., onconase, DNase I, Staphylococcal enterotoxin-A, pokeweed antiviral protein, gelonin, diphtheria toxin, *Pseudomonas* exotoxin, and *Pseudomonas* endotoxin.

In certain embodiments, a therapeutic agent may be an immunomodulator. An immunomodulator is an agent that when present, alters, suppresses or stimulates the body's immune system. Immunomodulators of use may include a cytokine, a stem cell growth factor, a lymphotoxin, a hematopoietic factor, a colony stimulating factor (CSF), an interferon (IFN), erythropoietin, thrombopoietin and a combination thereof. Specifically useful are lymphotoxins such as tumor necrosis factor (TNF), hematopoietic factors, such as interleukin (IL), colony stimulating factor, such as granulocyte-colony stimulating factor (G-CSF) or granulocyte macrophage-colony stimulating factor (GM-CSF), interferon, such as interferons-α, -β or -γ, and stem cell growth factor, such as that designated "S1 factor".

In various embodiments, the therapeutic agent may include one or more cytokines, such as lymphokines, monokines, growth factors and traditional polypeptide hormones. Included among the cytokines are growth hormones such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); placenta growth factor (PlGF), hepatic growth factor; prostaglandin, fibroblast growth factor; prolactin; placental lactogen, OB protein; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-21, IL-25, LIF, kit-ligand or FLT-3, angiostatin, thrombospondin, endostatin, tumor necrosis factor (TNF, such as TNF-α) and LT. Chemokines of use may include RANTES, MCAF, MIP1-alpha, MIP1-Beta and IP-10.

Anti-angiogenic agents include angiostatin, baculostatin, canstatin, maspin, anti-VEGF antibodies, anti-PlGF peptides and antibodies, anti-vascular growth factor antibodies, anti-Flk-1 antibodies, anti-Flt-1 antibodies and peptides, anti-Kras antibodies, anti-cMET antibodies, anti-MIF (macrophage migration-inhibitory factor) antibodies, laminin peptides, fibronectin peptides, plasminogen activator inhibitors, tissue metalloproteinase inhibitors, interferons, interleukin-12, IP-10, Gro-β, thrombospondin, 2-methoxyoestradiol, proliferin-related protein, carboxiamidotriazole, CM101, Marimastat, pentosan polysulphate, angiopoietin-2, interferon-alpha, herbimycin A, PNU145156E, 16K prolactin fragment, Linomide (roquinimex), thalidomide, pentoxifylline, genistein, TNP-470, endostatin, paclitaxel, accutin, angiostatin, cidofovir, vincristine, bleomycin, AGM-1470, platelet factor 4 or minocycline may be of use.

Other useful therapeutic agents may comprise oligonucleotides, especially antisense oligonucleotides that preferably are directed against oncogenes and oncogene products, such as bcl-2 or p53. A preferred form of therapeutic oligonucleotide is siRNA.

Diagnostic Agents

Diagnostic agents may be selected from the group consisting of a radionuclide, a radiological contrast agent, a paramagnetic ion, a metal, a fluorescent label, a chemiluminescent label, an ultrasound contrast agent and a photoactive agent. Such diagnostic agents are well known and any such known diagnostic agent may be used. Non-limiting examples of diagnostic agents may include a radionuclide such as ^{110}In, ^{111}In, ^{177}Lu, ^{18}F, ^{52}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{86}Y, ^{90}Y, ^{89}Zr, ^{94m}Tc, ^{94}Tc, ^{99m}Tc, ^{120}I, ^{123}I, ^{124}I, ^{125}I, ^{131}I, $^{154-158}$Gd, ^{32}P, ^{11}C, ^{13}N, ^{15}O, ^{186}Re, ^{188}Re, ^{51}Mn, ^{52m}Mn, ^{55}Co, ^{72}As, ^{75}Br, ^{76}Br, ^{82m}Rb, ^{83}Sr, or other gamma-, beta-, or positron-emitters. Paramagnetic ions of use may include chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) or erbium (III). Metal contrast agents may include lanthanum (III), gold (III), lead (II) or bismuth (III). Ultrasound contrast agents may comprise liposomes, such as gas filled liposomes. Radiopaque diagnostic agents may be selected from compounds, barium compounds, gallium compounds, and thallium compounds. A wide variety of fluorescent labels are known in the art, including but not limited to fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine. Chemiluminescent labels of use may include luminol, isoluminol, an aromatic acridinium ester, an imidazole, an acridinium salt or an oxalate ester.

Immunoconjugates

In certain embodiments, the anti-cancer vaccine DNL construct may be conjugated to one or more therapeutic or diagnostic agents. The therapeutic agents do not need to be the same but can be different, e.g. a drug and a radioisotope. For example, ^{131}I can be incorporated into a tyrosine of an antibody or fusion protein and a drug attached to an epsilon amino group of a lysine residue. Therapeutic and diagnostic agents also can be attached, for example to reduced SH groups and/or to carbohydrate side chains. Many methods for making covalent or non-covalent conjugates of therapeutic or diagnostic agents with antibodies or fusion proteins are known in the art and any such known method may be utilized.

A therapeutic or diagnostic agent can be attached at the hinge region of a reduced antibody component via disulfide bond formation. Alternatively, such agents can be attached using a heterobifunctional cross-linker, such as N-succinyl 3-(2-pyridyldithio)propionate (SPDP). Yu et al., *Int. J. Can-*

cer 56:244 (1994). General techniques for such conjugation are well-known in the art. See, for example, Wong, CHEMISTRY OF PROTEIN CONJUGATION AND CROSS-LINKING (CRC Press 1991); Upeslacis et al., "Modification of Antibodies by Chemical Methods," in MONOCLONAL ANTIBODIES: PRINCIPLES AND APPLICATIONS, Birch et al. (eds.), pages 187-230 (Wiley-Liss, Inc. 1995); Price, "Production and Characterization of Synthetic Peptide-Derived Antibodies," in MONOCLONAL ANTIBODIES: PRODUCTION, ENGINEERING AND CLINICAL APPLICATION, Ritter et al. (eds.), pages 60-84 (Cambridge University Press 1995). Alternatively, the therapeutic or diagnostic agent can be conjugated via a carbohydrate moiety in the Fc region of the antibody. The carbohydrate group can be used to increase the loading of the same agent that is bound to a thiol group, or the carbohydrate moiety can be used to bind a different therapeutic or diagnostic agent.

Methods for conjugating peptides to antibody components via an antibody carbohydrate moiety are well-known to those of skill in the art. See, for example, Shih et al., *Int. J. Cancer* 41:832 (1988); Shih et al., *Int. J. Cancer* 46:1101 (1990); and Shih et al., U.S. Pat. No. 5,057,313, incorporated herein in their entirety by reference. The general method involves reacting an antibody component having an oxidized carbohydrate portion with a carrier polymer that has at least one free amine function. This reaction results in an initial Schiff base (imine) linkage, which can be stabilized by reduction to a secondary amine to form the final conjugate.

The Fc region may be absent if the antibody used as the antibody component of the immunoconjugate is an antibody fragment. However, it is possible to introduce a carbohydrate moiety into the light chain variable region of a full length antibody or antibody fragment. See, for example, Leung et al., *J. Immunol.* 154:5919 (1995); Hansen et al., U.S. Pat. No. 5,443,953 (1995), Leung et al., U.S. Pat. No. 6,254,868, incorporated herein by reference in their entirety. The engineered carbohydrate moiety is used to attach the therapeutic or diagnostic agent.

In some embodiments, a chelating agent may be attached to an antibody, antibody fragment or fusion protein and used to chelate a therapeutic or diagnostic agent, such as a radionuclide. Exemplary chelators include but are not limited to DTPA (such as Mx-DTPA), DOTA, TETA, NETA or NOTA. Methods of conjugation and use of chelating agents to attach metals or other ligands to proteins are well known in the art (see, e.g., U.S. patent application Ser. No. 12/112, 289, incorporated herein by reference in its entirety).

In certain embodiments, radioactive metals or paramagnetic ions may be attached to proteins or peptides by reaction with a reagent having a long tail, to which may be attached a multiplicity of chelating groups for binding ions. Such a tail can be a polymer such as a polylysine, polysaccharide, or other derivatized or derivatizable chains having pendant groups to which can be bound chelating groups such as, e.g., ethylenediaminetetraacetic acid (EDTA), diethylenetri-aminepentaacetic acid (DTPA), porphyrins, polyamines, crown ethers, bis-thiosemicarbazones, polyoximes, and like groups known to be useful for this purpose.

Chelates may be directly linked to antibodies or peptides, for example as disclosed in U.S. Pat. No. 4,824,659, incorporated herein in its entirety by reference. Particularly useful metal-chelate combinations include 2-benzyl-DTPA and its monomethyl and cyclohexyl analogs, used with diagnostic isotopes in the general energy range of 60 to 4,000 keV, such as ^{125}I, ^{131}I, ^{123}I, ^{124}I, ^{62}Cu, ^{64}Cu, ^{18}F, ^{111}In, ^{67}Ga, ^{68}Ga,

^{99m}Tc, ^{94m}Tc, ^{11}C, ^{13}N, ^{15}O, ^{76}Br, for radioimaging. The same chelates, when complexed with non-radioactive metals, such as manganese, iron and gadolinium are useful for MRI. Macrocyclic chelates such as NOTA, DOTA, and TETA are of use with a variety of metals and radiometals, most particularly with radionuclides of gallium, yttrium and copper, respectively. Such metal-chelate complexes can be made very stable by tailoring the ring size to the metal of interest. Other ring-type chelates such as macrocyclic polyethers, which are of interest for stably binding nuclides, such as ^{223}Ra for RAIT are encompassed.

More recently, methods of ^{18}F-labeling of use in PET scanning techniques have been disclosed, for example by reaction of F-18 with a metal or other atom, such as aluminum. The ^{18}F—Al conjugate may be complexed with chelating groups, such as DOTA, NOTA or NETA that are attached directly to antibodies or used to label targetable constructs in pre-targeting methods. Such F-18 labeling techniques are disclosed in U.S. patent application Ser. No. 12/112,289, filed Apr. 30, 2008, the entire text of which is incorporated herein by reference.

Methods of Therapeutic Treatment

Various embodiments concern methods of treating a cancer, such as multiple myeloma, in a subject, such as a mammal, including humans, domestic or companion pets, such as dogs and cats. The methods may comprise administering to a subject a therapeutically effective amount of an anti-cancer vaccine DNL construct. In preferred embodiments, the anti-cancer vaccine DNL construct comprises an anti-CD74 antibody or fragment thereof and a CD20 xeno-antigen, as described in further detail in the Examples below.

The administration of anti-cancer vaccine DNL construct can be supplemented by administering concurrently or sequentially a therapeutically effective amount of another antibody that binds to or is reactive with another antigen on the surface of the target cell. Preferred additional MAbs comprise at least one humanized, chimeric or human MAb selected from the group consisting of a MAb reactive with CD209 (DC-SIGN), CD34, CD74, CD205, TLR 2 (toll-like receptor 2), TLR 4, TLR 7, TLR 9, BDCA-2, BDCA-3, BDCA-4 and HLA-DR. Various antibodies of use are known to those of skill in the art, as discussed above. See, for example, Ghetie et al., *Cancer Res.* 48:2610 (1988); Hekman et al., *Cancer Immunol. Immunother.* 32:364 (1991); Longo, *Curr. Opin. Oncol.* 8:353 (1996), U.S. Pat. Nos. 5,798,554; 6,187,287; 6,306,393; 6,676,924; 7,109,304; 7,151,164; 7,230,084; 7,230,085; 7,238,785; 7,238,786; 7,282,567; 7,300,655; 7,312,318; and U.S. Patent Application Publ. Nos. 20080131363; 20080089838; 20070172920; 20060193865; 20060210475; 20080138333; and 20080146784, the Examples section of each cited patent or application incorporated herein by reference.

In alternative embodiments an antibody or fragment thereof against another dendritic cell antigen, such as CD209 (DC-SIGN), CD34, CD205, TLR 2 (toll-like receptor 2), TLR 4, TLR 7, TLR 9, BDCA-2, BDCA-3, BDCA-4 or HLA-DR, may be substituted for the anti-CD74 antibody in the DNL complex. Such antibodies may be obtained from public sources like the American Type Culture Collection or from commercial antibody vendors. For example, antibodies against CD209(DC-SIGN), CD34, BDCA-2, TLR2, TLR 4, TLR 7 and TLR 9 may be purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). Antibodies against CD205 and BDCA-3 may be purchased from Miltenyi Biotec Inc. (Auburn, Calif.). Numerous other commercial sources of antibodies are known to the skilled artisan.

The anti-cancer vaccine DNL construct therapy can be further supplemented with the administration, either concurrently or sequentially, of at least one therapeutic agent. Therapeutic agents used for the treatment of multiple myeloma include dexamethasone, thalidomide/dexamethasone, cyclophosphamide, VAD (vincristine, doxorubicin and dexamethasone), DVd (DOXIL® (PEGylated doxorubicin), vincristine and reduced schedule dexamethasone), BCNU, melphalan, carmustine, bortezomib (VELCADE®), prednisone and corticosteroids. The individual therapeutic agents may be used alone or in various combinations known in the art, such as CP (cyclophosphamide, prednisone), CT (cyclophosphamide, thalidomide), VBMCP (vincristine, BCNU, melphalan, cyclophosphamide, melphalan), VMCP (vincristine, melphalan, cyclophosphamide, prednisone), DT-PACE (dexamethasone, thalidomide, cisplatin, doxorubicin, cyclophosphamide, etoposide), MPT (melphalan, prednisone, thalidomide), CVAD (cyclophosphamide and VAD), EDAP (etoposide, dexamethasone, ara-C, cisplatin) MTD (melphalan, thalidomide, dexamethasone), VT (VELCADE®, thalidomide), VDT (VELCADE®, doxorubicin, thalidomide), VADT (VELCADE®, adriamycin, thalidomide, dexamethasone) or DCEP (dexamethasone, cyclophosphamide, etoposide, cisplatin).

Chemotherapeutic treatment of multiple myeloma prior to stem cell transplantation is referred to as induction therapy. Certain of the chemotherapeutic agents listed herein are more suitable for induction therapy than others. Examples of chemotherapeutic treatments of use for induction therapy for MM include dexamethasone, thalidomide/dexamethasone, cyclophosphamide, VAD and DVd. Because MM is often resistant to chemotherapeutic treatment, administration of therapeutic agents may occur at higher doses than are used in conventional chemotherapy. Such high-dose chemotherapy usually results in bone marrow toxicity and is often used in conjunction with stem cell transplantation. Dosages and schedules for chemotherapeutic treatment of MM are well known in the art and any such known dosage and/or schedule may be utilized in conjunction with administration of the anti-cancer vaccine DNL construct.

Where the DNL vaccine is used for other types of cancers besides MM, other chemotherapeutic regimens are known. For example, "CVB" (1.5 g/m^2 cyclophosphamide, 200-400 mg/m^2 etoposide, and 150-200 mg/m^2 carmustine) is a regimen used to treat non-Hodgkin's lymphoma. Patti et al., *Eur. J. Haematol.* 51:18 (1993). Other suitable combination chemotherapeutic regimens are well-known to those of skill in the art. See, for example, Freedman et al., "Non-Hodgkin's Lymphomas," in CANCER MEDICINE, VOLUME 2, 3rd Edition, Holland et al. (eds.), pages 2028-2068 (Lea & Febiger 1993). As an illustration, first generation chemotherapeutic regimens for treatment of intermediate-grade non-Hodgkin's lymphoma (NHL) include C-MOPP (cyclophosphamide, vincristine, procarbazine and prednisone) and CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone). A useful second generation chemotherapeutic regimen is m-BACOD (methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, dexamethasone and leucovorin), while a suitable third generation regimen is MACOP-B (methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin and leucovorin). Chemotherapeutic agents of use against other types of cancers include, but are not limited to, 5-fluorouracil, aplidin, azaribine, anastrozole, anthracyclines, bendamustine, bleomycin, bortezomib, bryostatin-1, busulfan, calicheamycin, camptothecin, carboplatin, 10-hydroxycamptothecin, carmustine, celebrex, chlorambucil, cisplatin (CDDP), Cox-2

inhibitors, irinotecan (CPT-11), SN-38, carboplatin, cladribine, camptothecans, cyclophosphamide, cytarabine, dacarbazine, docetaxel, dactinomycin, daunorubicin, doxorubicin, 2-pyrrolinodoxorubicine (2P-DOX), cyano-morpholino doxorubicin, doxorubicin glucuronide, epirubicin glucuronide, estramustine, epidophyllotoxin, estrogen receptor binding agents, etoposide (VP16), etoposide glucuronide, etoposide phosphate, floxuridine (FUdR), 3',5'-O-dioleoyl-FudR (FUdR-dO), fludarabine, flutamide, farnesyl-protein transferase inhibitors, gemcitabine, hydroxyurea, idarubicin, ifosfamide, L-asparaginase, lenolidamide, leucovorin, lomustine, mechlorethamine, melphalan, mercaptopurine, 6-mercaptopurine, methotrexate, mitoxantrone, mithramycin, mitomycin, mitotane, navelbine, nitrosurea, phenyl butyrate, plicomycin, procarbazine, paclitaxel, pentostatin, PSI-341, raloxifene, semustine, streptozocin, tamoxifen, taxol, temazolomide (an aqueous form of DTIC), transplatinum, thalidomide, thioguanine, thiotepa, teniposide, topotecan, uracil mustard, vinorelbine, vinblastine, vincristine and vinca alkaloids.

Formulations

The anti-cancer vaccine DNL construct can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the anti-cancer vaccine DNL construct is combined in a mixture with a pharmaceutically suitable excipient. Sterile phosphate-buffered saline is one example of a pharmaceutically suitable excipient. Other suitable excipients are well-known to those in the art. See, for example, Ansel et al., PHARMACEUTICAL DOSAGE FORMS AND DRUG DELIVERY SYSTEMS, 5th Edition (Lea & Febiger 1990), and Gennaro (ed.), REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Edition (Mack Publishing Company 1990), and revised editions thereof.

The anti-cancer vaccine can be formulated for intravenous administration via, for example, bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions can take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient can be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

Additional pharmaceutical methods may be employed to control the duration of action of the anti-cancer vaccine. Control release preparations can be prepared through the use of polymers to complex or adsorb the anti-cancer vaccine DNL construct. For example, biocompatible polymers include matrices of poly(ethylene-co-vinyl acetate) and matrices of a polyanhydride copolymer of a stearic acid dimer and sebacic acid. Sherwood et al., *Bio/Technology* 10:1446 (1992). The rate of release from such a matrix depends upon the molecular weight of the anti-cancer vaccine DNL construct, the amount of anti-cancer vaccine within the matrix, and the size of dispersed particles. Saltzman et al., *Biophys. J.* 55:163 (1989); Sherwood et al., supra. Other solid dosage forms are described in Ansel et al., PHARMACEUTICAL DOSAGE FORMS AND DRUG DELIVERY SYSTEMS, 5th Edition (Lea & Febiger 1990), and Gennaro (ed.), REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Edition (Mack Publishing Company 1990), and revised editions thereof.

The anti-cancer vaccine DNL construct may also be administered to a mammal subcutaneously or even by other parenteral routes. Moreover, the administration may be by continuous infusion or by single or multiple boluses. Pref-

erably, the anti-cancer vaccine is administered as a single or multiple boluses via subcutaneous injection.

Generally, the dosage of an administered anti-cancer vaccine DNL construct for humans will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition and previous medical history. It may be desirable to provide the recipient with a dosage of anti-cancer vaccine DNL construct that is in the range of from about 1 mg/kg to 25 mg/kg as a single administration, although a lower or higher dosage also may be administered as circumstances dictate. A dosage of 1-20 mg/kg for a 70 kg patient, for example, is 70-1,400 mg, or 41-824 mg/m^2 for a 1.7-m patient. The dosage may be repeated as needed for induction of an immune response.

In preferred embodiments, the vaccine DNL constructs are of use for therapy of cancer. Examples of cancers include, but are not limited to, carcinoma, lymphoma, glioblastoma, melanoma, sarcoma, and leukemia, myeloma, or lymphoid malignancies. More particular examples of such cancers are noted below and include: squamous cell cancer (e.g., epithelial squamous cell cancer), Ewing sarcoma, Wilms tumor, astrocytomas, lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma multiforme, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, hepatocellular carcinoma, neuroendocrine tumors, medullary thyroid cancer, differentiated thyroid carcinoma, breast cancer, ovarian cancer, colon cancer, rectal cancer, endometrial cancer or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulvar cancer, anal carcinoma, penile carcinoma, as well as head-and-neck cancer. The term "cancer" includes primary malignant cells or tumors (e.g., those whose cells have not migrated to sites in the subject's body other than the site of the original malignancy or tumor) and secondary malignant cells or tumors (e.g., those arising from metastasis, the migration of malignant cells or tumor cells to secondary sites that are different from the site of the original tumor).

Other examples of cancers or malignancies include, but are not limited to: Acute Childhood Lymphoblastic Leukemia, Acute Lymphoblastic Leukemia, Acute Lymphocytic Leukemia, Acute Myeloid Leukemia, Adrenocortical Carcinoma, Adult (Primary) Hepatocellular Cancer, Adult (Primary) Liver Cancer, Adult Acute Lymphocytic Leukemia, Adult Acute Myeloid Leukemia, Adult Hodgkin's Lymphoma, Adult Lymphocytic Leukemia, Adult Non-Hodgkin's Lymphoma, Adult Primary Liver Cancer, Adult Soft Tissue Sarcoma, AIDS-Related Lymphoma, AIDS-Related Malignancies, Anal Cancer, Astrocytoma, Bile Duct Cancer, Bladder Cancer, Bone Cancer, Brain Stem Glioma, Brain Tumors, Breast Cancer, Cancer of the Renal Pelvis and Ureter, Central Nervous System (Primary) Lymphoma, Central Nervous System Lymphoma, Cerebellar Astrocytoma, Cerebral Astrocytoma, Cervical Cancer, Childhood (Primary) Hepatocellular Cancer, Childhood (Primary) Liver Cancer, Childhood Acute Lymphoblastic Leukemia, Childhood Acute Myeloid Leukemia, Childhood Brain Stem Glioma, Childhood Cerebellar Astrocytoma, Childhood Cerebral Astrocytoma, Childhood Extracranial Germ Cell Tumors, Childhood Hodgkin's Disease, Childhood Hodgkin's Lymphoma, Childhood Hypothalamic and Visual Pathway Glioma, Childhood Lymphoblastic Leukemia, Childhood Medulloblastoma, Childhood Non-Hodgkin's Lymphoma, Childhood Pineal and Supratentorial Primitive Neuroectodermal Tumors, Childhood Primary Liver Cancer, Childhood Rhabdomyosarcoma, Childhood Soft Tissue Sarcoma, Childhood Visual Pathway and Hypothalamic Glioma, Chronic Lymphocytic Leukemia, Chronic Myelogenous Leukemia, Colon Cancer, Cutaneous T-Cell Lymphoma, Endocrine Pancreas Islet Cell Carcinoma, Endometrial Cancer, Ependymoma, Epithelial Cancer, Esophageal Cancer, Ewing's Sarcoma and Related Tumors, Exocrine Pancreatic Cancer, Extracranial Germ Cell Tumor, Extragonadal Germ Cell Tumor, Extrahepatic Bile Duct Cancer, Eye Cancer, Female Breast Cancer, Gaucher's Disease, Gallbladder Cancer, Gastric Cancer, Gastrointestinal Carcinoid Tumor, Gastrointestinal Tumors, Germ Cell Tumors, Gestational Trophoblastic Tumor, Hairy Cell Leukemia, Head and Neck Cancer, Hepatocellular Cancer, Hodgkin's Lymphoma, Hypergammaglobulinemia, Hypopharyngeal Cancer, Intestinal Cancers, Intraocular Melanoma, Islet Cell Carcinoma, Islet Cell Pancreatic Cancer, Kaposi's Sarcoma, Kidney Cancer, Laryngeal Cancer, Lip and Oral Cavity Cancer, Liver Cancer, Lung Cancer, Lymphoproliferative Disorders, Macroglobulinemia, Male Breast Cancer, Malignant Mesothelioma, Malignant Thymoma, Medulloblastoma, Melanoma, Mesothelioma, Metastatic Occult Primary Squamous Neck Cancer, Metastatic Primary Squamous Neck Cancer, Metastatic Squamous Neck Cancer, Multiple Myeloma, Multiple Myeloma/Plasma Cell Neoplasm, Myelodysplastic Syndrome, Myelogenous Leukemia, Myeloid Leukemia, Myeloproliferative Disorders, Nasal Cavity and Paranasal Sinus Cancer, Nasopharyngeal Cancer, Neuroblastoma, Non-Hodgkin's Lymphoma, Nonmelanoma Skin Cancer, Non-Small Cell Lung Cancer, Occult Primary Metastatic Squamous Neck Cancer, Oropharyngeal Cancer, Osteo-/Malignant Fibrous Sarcoma, Osteosarcoma/Malignant Fibrous Histiocytoma, Osteosarcoma/Malignant Fibrous Histiocytoma of Bone, Ovarian Epithelial Cancer, Ovarian Germ Cell Tumor, Ovarian Low Malignant Potential Tumor, Pancreatic Cancer, Paraproteinemias, Polycythemia vera, Parathyroid Cancer, Penile Cancer, Pheochromocytoma, Pituitary Tumor, Primary Central Nervous System Lymphoma, Primary Liver Cancer, Prostate Cancer, Rectal Cancer, Renal Cell Cancer, Renal Pelvis and Ureter Cancer, Retinoblastoma, Rhabdomyosarcoma, Salivary Gland Cancer, Sarcoidosis Sarcomas, Sezary Syndrome, Skin Cancer, Small Cell Lung Cancer, Small Intestine Cancer, Soft Tissue Sarcoma, Squamous Neck Cancer, Stomach Cancer, Supratentorial Primitive Neuroectodermal and Pineal Tumors, T-Cell Lymphoma, Testicular Cancer, Thymoma, Thyroid Cancer, Transitional Cell Cancer of the Renal Pelvis and Ureter, Transitional Renal Pelvis and Ureter Cancer, Trophoblastic Tumors, Ureter and Renal Pelvis Cell Cancer, Urethral Cancer, Uterine Cancer, Uterine Sarcoma, Vaginal Cancer, Visual Pathway and Hypothalamic Glioma, Vulvar Cancer, Waldenstrom's Macroglobulinemia, Wilms' Tumor, and any other hyperproliferative disease, besides neoplasia, located in an organ system listed above.

The methods and compositions described and claimed herein may be used to treat malignant or premalignant conditions and to prevent progression to a neoplastic or malignant state, including but not limited to those disorders described above. Such uses are indicated in conditions known or suspected of preceding progression to neoplasia or cancer, in particular, where non-neoplastic cell growth consisting of hyperplasia, metaplasia, or most particularly, dysplasia has occurred (for review of such abnormal growth conditions, see Robbins and Angell, Basic Pathology, 2d Ed., W. B. Saunders Co., Philadelphia, pp. 68-79 (1976)).

Dysplasia is frequently a forerunner of cancer, and is found mainly in the epithelia. It is the most disorderly form of non-neoplastic cell growth, involving a loss in individual cell uniformity and in the architectural orientation of cells. Dysplasia characteristically occurs where there exists chronic irritation or inflammation. Dysplastic disorders which can be treated include, but are not limited to, anhidrotic ectodermal dysplasia, anterofacial dysplasia, asphyxiating thoracic dysplasia, atriodigital dysplasia, bronchopulmonary dysplasia, cerebral dysplasia, cervical dysplasia, chondroectodermal dysplasia, cleidocranial dysplasia, congenital ectodermal dysplasia, craniodiaphysial dysplasia, craniocarpotarsal dysplasia, craniometaphysial dysplasia, dentin dysplasia, diaphysial dysplasia, ectodermal dysplasia, enamel dysplasia, encephalo-ophthalmic dysplasia, dysplasia epiphysialis hemimelia, dysplasia epiphysialis multiplex, dysplasia epiphysialis punctata, epithelial dysplasia, faciodigitogenital dysplasia, familial fibrous dysplasia of jaws, familial white folded dysplasia, fibromuscular dysplasia, fibrous dysplasia of bone, florid osseous dysplasia, hereditary renal-retinal dysplasia, hidrotic ectodermal dysplasia, hypohidrotic ectodermal dysplasia, lymphopenic thymic dysplasia, mammary dysplasia, mandibulofacial dysplasia, metaphysial dysplasia, Mondini dysplasia, monostotic fibrous dysplasia, mucoepithelial dysplasia, multiple epiphysial dysplasia, oculoauriculovertebral dysplasia, oculodentodigital dysplasia, oculovertebral dysplasia, odontogenic dysplasia, opthalmomandibulomelic dysplasia, periapical cemental dysplasia, polyostotic fibrous dysplasia, pseudoachondroplastic spondyloepiphysial dysplasia, retinal dysplasia, septo-optic dysplasia, spondyloepiphysial dysplasia, and ventriculoradial dysplasia.

Additional pre-neoplastic disorders which can be treated include, but are not limited to, benign dysproliferative disorders (e.g., benign tumors, fibrocystic conditions, tissue hypertrophy, intestinal polyps or adenomas, and esophageal dysplasia), leukoplakia, keratoses, Bowen's disease, Farmer's Skin, solar cheilitis, and solar keratosis.

In preferred embodiments, the method of the invention is used to inhibit growth, progression, and/or metastasis of cancers, in particular those listed above.

Additional hyperproliferative diseases, disorders, and/or conditions include, but are not limited to, progression, and/or metastases of malignancies and related disorders such as leukemia (including acute leukemias (e.g., acute lymphocytic leukemia, acute myelocytic leukemia (including myeloblastic, promyelocytic, myelomonocytic, monocytic, and erythroleukemia)) and chronic leukemias (e.g., chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia)), polycythemia vera, lymphomas (e.g., Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors including, but not limited to, sarcomas and carcinomas such as fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, testicular tumor, lung carcinoma, small cell

lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, emangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, and retinoblastoma.

Kits

Various embodiments may concern kits containing components suitable for treating or diagnosing diseased tissue in a patient. Exemplary kits may contain at least one or more anti-cancer vaccine constructs as described herein. If the composition containing components for administration is not formulated for delivery via the alimentary canal, such as by oral delivery, a device capable of delivering the kit components through some other route may be included. One type of device, for applications such as parenteral delivery, is a syringe that is used to inject the composition into the body of a subject. Inhalation devices may also be used. In certain embodiments, a therapeutic agent may be provided in the form of a prefilled syringe or autoinjection pen containing a sterile, liquid formulation or lyophilized preparation.

The kit components may be packaged together or separated into two or more containers. In some embodiments, the containers may be vials that contain sterile, lyophilized formulations of a composition that are suitable for reconstitution. A kit may also contain one or more buffers suitable for reconstitution and/or dilution of other reagents. Other containers that may be used include, but are not limited to, a pouch, tray, box, tube, or the like. Kit components may be packaged and maintained sterilely within the containers. Another component that can be included is instructions to a person using a kit for its use.

Expression Vectors

Still other embodiments may concern DNA sequences comprising a nucleic acid encoding a anti-cancer vaccine construct, or its constituent fusion proteins. Fusion proteins may comprise an anti-CD74 antibody or CD20 xenoantigen attached to a different peptide or protein, such as the AD and DDD peptides utilized for DNL construct formation as discussed in more detail in the Examples below. Alternatively the encoded fusion proteins may comprise a DDD or AD moiety attached to a different antibody or xenoantigen.

Various embodiments relate to expression vectors comprising the coding DNA sequences. The vectors may contain sequences encoding the light and heavy chain constant regions and the hinge region of a human immunoglobulin to which may be attached chimeric, humanized or human variable region sequences. The vectors may additionally contain promoters that express the encoded protein(s) in a selected host cell, enhancers and signal or leader sequences. Vectors that are particularly useful are pdHL2 or GS. More preferably, the light and heavy chain constant regions and hinge region may be from a human EU myeloma immunoglobulin, where optionally at least one of the amino acid in the allotype positions is changed to that found in a different IgG1 allotype, and wherein optionally amino acid 253 of the heavy chain of EU based on the EU number system may be replaced with alanine. See Edelman et al., *Proc. Natl. Acad. Sci USA* 63:78-85 (1969). In other embodiments, an IgG1 sequence may be converted to an IgG4 sequence.

The skilled artisan will realize that methods of genetically engineering expression constructs and insertion into host cells to express engineered proteins are well known in the art and a matter of routine experimentation. Host cells and methods of expression of cloned antibodies or fragments have been described, for example, in U.S. patent application Ser. No. 11/187,863, filed Jul. 25, 2005; Ser. No. 11/253,666,

filed Oct. 20, 2005 and Ser. No. 11/487,215, filed Jul. 14, 2006, the Examples section of each incorporated herein by reference.

EXAMPLES

The following examples are provided to illustrate, but not to limit, the claims of the present invention.

Example 1

Preparation of Dock-and-Lock (DNL) Constructs

DDD and AD Fusion Proteins

The DNL technique can be used to make dimers, trimers, tetramers, hexamers, etc. comprising virtually any antibodies or fragments thereof or other effector moieties. For certain preferred embodiments, IgG antibodies, F(ab')$_2$ antibody fragments and xenoantigens, such as CD20 xenoantigens, may be produced as fusion proteins containing either a dimerization and docking domain (DDD) or anchoring domain (AD) sequence. Although in preferred embodiments the DDD and AD moieties are produced as fusion proteins, the skilled artisan will realize that other methods of conjugation, such as chemical cross-linking, may be utilized within the scope of the claimed methods and compositions.

DNL constructs may be formed by combining, for example, an Fab-DDD fusion protein of an anti-CD74 antibody with a CD20-AD fusion protein. Alternatively, constructs may be made that combine IgG-AD fusion proteins with CD20-DDD fusion proteins. The technique is not limiting and any protein or peptide of use may be produced as an AD or DDD fusion protein for incorporation into a DNL construct. Where chemical cross-linking is utilized, the AD and DDD conjugates are not limited to proteins or peptides and may comprise any molecule that may be cross-linked to an AD or DDD sequence using any cross-linking technique known in the art.

Independent transgenic cell lines may be developed for each DDD or AD fusion protein. Once produced, the modules can be purified if desired or maintained in the cell culture supernatant fluid. Following production, any DDD-fusion protein module can be combined with any AD-fusion protein module to generate a DNL construct. For different types of constructs, different AD or DDD sequences may be utilized. Exemplary DDD and AD sequences are provided below.

```
                                          (SEQ ID NO: 10)
DDD1:  SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA

                                          (SEQ ID NO: 11)
DDD2:  CGHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA

                                          (SEQ ID NO: 12)
AD1:   QIEYLAKQIVDNAIQQA

                                          (SEQ ID NO: 13)
AD2:   CGQIEYLAKQIVDNAIQQAGC
```

Expression Vectors

The plasmid vector pdHL2 has been used to produce a number of antibodies and antibody-based constructs. See Gillies et al., J Immunol Methods (1989), 125:191-202; Losman et al., Cancer (Phila) (1997), 80:2660-6. The dicistronic mammalian expression vector directs the synthesis of the heavy and light chains of IgG. The vector sequences are mostly identical for many different IgG-pdHL2 constructs, with the only differences existing in the variable domain (VH and VL) sequences. Using molecular biology

tools known to those skilled in the art, these IgG expression vectors can be converted into Fab-DDD or Fab-AD expression vectors. To generate Fab-DDD expression vectors, the coding sequences for the hinge, CH2 and CH3 domains of the heavy chain are replaced with a sequence encoding the first 4 residues of the hinge, a 14 residue Gly-Ser linker and the first 44 residues of human RIIα (referred to as DDD1). To generate Fab-AD expression vectors, the sequences for the hinge, CH2 and CH3 domains of IgG are replaced with a sequence encoding the first 4 residues of the hinge, a 15 residue Gly-Ser linker and a 17 residue synthetic AD called AKAP-IS (referred to as AD1), which was generated using bioinformatics and peptide array technology and shown to bind RIIα dimers with a very high affinity (0.4 nM). See Alto, et al. Proc. Natl. Acad. Sci., U.S.A (2003), 100:4445-50.

Two shuttle vectors were designed to facilitate the conversion of IgG-pdHL2 vectors to either Fab-DDD1 or Fab-AD1 expression vectors, as described below.

Preparation of CH1

The CH1 domain was amplified by PCR using the pdHL2 plasmid vector as a template. The left PCR primer consisted of the upstream (5') end of the CH1 domain and a SacII restriction endonuclease site, which is 5' of the CH1 coding sequence. The right primer consisted of the sequence coding for the first 4 residues of the hinge (PKSC (SEQ ID NO:29) followed by four glycines and a serine, with the final two codons (GS) comprising a Bam HI restriction site. The 410 bp PCR amplimer was cloned into the PGEMT® PCR cloning vector (PROMEGA®, Inc.) and clones were screened for inserts in the T7 (5') orientation.

Construction of (G$_4$S)$_2$DDD1 ((G$_4$S)$_2$ Disclosed as SEQ ID NO:14)

A duplex oligonucleotide, designated (G$_4$S)$_2$DDD1 ((G$_4$S)$_2$ disclosed as SEQ ID NO:14), was synthesized by Sigma GENOSYS® (Haverhill, UK) to code for the amino acid sequence of DDD1 preceded by 11 residues of the linker peptide, with the first two codons comprising a BamHI restriction site. A stop codon and an EagI restriction site are appended to the 3' end. The encoded polypeptide sequence is shown below.

```
                                       (SEQ ID NO: 15)
GSGGGGSGGGGSHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTR
LREARA
```

Two oligonucleotides, designated RIIA1-44 top and RIIA1-44 bottom, that overlap by 30 base pairs on their 3' ends, were synthesized (Sigma GENOSYS®) and combined to comprise the central 154 base pairs of the 174 bp DDD1 sequence. The oligonucleotides were annealed and subjected to a primer extension reaction with Taq polymerase. Following primer extension, the duplex was amplified by PCR. The amplimer was cloned into PGEMT® and screened for inserts in the T7 (5') orientation.

Construction of (G$_4$S)$_2$-AD1 ((G$_4$S)$_2$ Disclosed as SEQ ID NO:14)

A duplex oligonucleotide, designated (G$_4$S)$_2$-AD1 ((G$_4$S)$_2$ disclosed as SEQ ID NO:14), was synthesized (Sigma GENOSYS®) to code for the amino acid sequence of AD1 preceded by 11 residues of the linker peptide with the first two codons comprising a BamHI restriction site. A stop codon and an EagI restriction site are appended to the 3' end. The encoded polypeptide sequence is shown below.

```
GSGGGGSGGGGSQIEYLAKQIVDNAIQQA      (SEQ ID NO: 16)
```

Two complimentary overlapping oligonucleotides encoding the above peptide sequence, designated AKAP-IS Top and AKAP-IS Bottom, were synthesized and annealed. The duplex was amplified by PCR. The amplimer was cloned into the PGEMT® vector and screened for inserts in the T7 (5') orientation.

Ligating DDD1 with CH1

A 190 bp fragment encoding the DDD1 sequence was excised from PGEMT® with BamHI and NotI restriction enzymes and then ligated into the same sites in CH1-PGEMT® to generate the shuttle vector CH1-DDD1-PGEMT®.

Ligating AD1 with CH1

A 110 bp fragment containing the AD1 sequence was excised from PGEMT® with BamHI and NotI and then ligated into the same sites in CH1-PGEMT® to generate the shuttle vector CH1-AD1-PGEMT®.

Cloning CH1-DDD1 or CH1-AD1 into pdHL2-Based Vectors

With this modular design either CH1-DDD1 or CH1-AD1 can be incorporated into any IgG construct in the pdHL2 vector. The entire heavy chain constant domain is replaced with one of the above constructs by removing the SacII/EagI restriction fragment (CH1-CH3) from pdHL2 and replacing it with the SacII/EagI fragment of CH1-DDD1 or CH1-AD1, which is excised from the respective pGemT shuttle vector.

Construction of h679-Fd-AD1-pdHL2

h679-Fd-AD1-pdHL2 is an expression vector for production of h679 Fab with AD1 coupled to the carboxyl terminal end of the CH1 domain of the Fd via a flexible Gly/Ser peptide spacer composed of 14 amino acid residues. A pdHL2-based vector containing the variable domains of h679 was converted to h679-Fd-AD1-pdHL2 by replacement of the SacII/EagI fragment with the CH1-AD1 fragment, which was excised from the CH1-AD1-SV3 shuttle vector with SacII and EagI.

Construction of C-DDD1-Fd-hMN-14-pdHL2

C-DDD1-Fd-hMN-14-pdHL2 is an expression vector for production of a stable dimer that comprises two copies of a fusion protein C-DDD1-Fab-hMN-14, in which DDD1 is linked to hMN-14 Fab at the carboxyl terminus of CH1 via a flexible peptide spacer. The plasmid vector hMN-14(I)-pdHL2, which has been used to produce hMN-14 IgG, was converted to C-DDD1-Fd-hMN-14-pdHL2 by digestion with SacII and EagI restriction endonucleases to remove the CH1-CH3 domains and insertion of the CH1-DDD1 fragment, which was excised from the CH1-DDD1-SV3 shuttle vector with SacII and EagI.

The same technique has been utilized to produce plasmids for Fab expression of a wide variety of known antibodies, such as hLL1, hLL2, hPAM4, hR1, hRS7, hMN-14, hMN-15, hA19, hA20 and many others. Generally, the antibody variable region coding sequences were present in a pdHL2 expression vector and the expression vector was converted for production of an AD- or DDD-fusion protein as described above.

Construction of C-DDD2-Fd-hMN-14-pdHL2

C-DDD2-Fd-hMN-14-pdHL2 is an expression vector for production of C-DDD2-Fab-hMN-14, which possesses a dimerization and docking domain sequence of DDD2 appended to the carboxyl terminus of the Fd of hMN-14 via a 14 amino acid residue Gly/Ser peptide linker. The fusion protein secreted is composed of two identical copies of hMN-14 Fab held together by non-covalent interaction of the DDD2 domains.

The expression vector was engineered as follows. Two overlapping, complimentary oligonucleotides, which comprise the coding sequence for part of the linker peptide (GGGGSGGGCG, SEQ ID NO:17) and residues 1-13 of DDD2, were made synthetically. The oligonucleotides were annealed and phosphorylated with T4 PNK, resulting in overhangs on the 5' and 3' ends that are compatible for ligation with DNA digested with the restriction endonucleases BamHI and PstI, respectively.

The duplex DNA was ligated with the shuttle vector CH1-DDD1-PGEMT®, which was prepared by digestion with BamHI and PstI, to generate the shuttle vector CH1-DDD2-PGEMT®. A 507 bp fragment was excised from CH1-DDD2-PGEMT® with SacII and EagI and ligated with the IgG expression vector hMN-14(I)-pdHL2, which was prepared by digestion with SacII and EagI. The final expression construct was designated C-DDD2-Fd-hMN-14-pdHL2. Similar techniques have been utilized to generated DDD2-fusion proteins of the Fab fragments of a number of different humanized antibodies.

Construction of h679-Fd-AD2-pdHL2

h679-Fd-AD2-pdHL2 is an expression vector for the production of h679-Fab-AD2, which possesses an anchoring domain sequence of AD2 appended to the carboxyl terminal end of the CH1 domain via a 14 amino acid residue Gly/Ser peptide linker. AD2 has one cysteine residue preceding and another one following the anchor domain sequence of AD1.

The expression vector was engineered as follows. Two overlapping, complimentary oligonucleotides which comprise the coding sequence for AD2 and part of the linker sequence, were made synthetically. The oligonucleotides were annealed and phosphorylated with T4 PNK, resulting in overhangs on the 5' and 3' ends that are compatible for ligation with DNA digested with the restriction endonucleases BamHI and SpeI, respectively.

The duplex DNA was ligated into the shuttle vector CH1-AD1-PGEMT®, which was prepared by digestion with BamHI and SpeI, to generate the shuttle vector CH1-AD2-PGEMT®. A 429 base pair fragment containing CH1 and AD2 coding sequences was excised from the shuttle vector with SacII and EagI restriction enzymes and ligated into h679-pdHL2 vector that prepared by digestion with those same enzymes. The final expression vector is h679-Fd-AD2-pdHL2.

Generation of TF2 Trimeric DNL Construct

A trimeric DNL construct designated TF2 was obtained by reacting C-DDD2-Fab-hMN-14 with h679-Fab-AD2. A pilot batch of TF2 was generated with >90% yield as follows. Protein L-purified C-DDD2-Fab-hMN-14 (200 mg) was mixed with h679-Fab-AD2 (60 mg) at a 1.4:1 molar ratio. The total protein concentration was 1.5 mg/ml in PBS containing 1 mM EDTA. Subsequent steps involved TCEP reduction, HIC chromatography, DMSO oxidation, and IMP 291 affinity chromatography. Before the addition of TCEP, SE-HPLC did not show any evidence of a_2b formation. Addition of 5 mM TCEP rapidly resulted in the formation of a_2b complex consistent with a 157 kDa protein expected for the binary structure. TF2 was purified to near homogeneity by IMP 291 affinity chromatography (not shown). IMP 291 is a synthetic peptide containing the HSG hapten to which the 679 Fab binds (Rossi et al., 2005, Clin Cancer Res 11:7122s-29s). SE-HPLC analysis of the IMP 291 unbound fraction demonstrated the removal of a_4, a_2 and free kappa chains from the product (not shown).

Non-reducing SDS-PAGE analysis demonstrated that the majority of TF2 exists as a large, covalent structure with a relative mobility near that of IgG (not shown). Reducing

SDS-PAGE shows that any additional bands apparent in the non-reducing gel are product-related (not shown), as only bands representing the constituent polypeptides of TF2 were evident (not shown). However, the relative mobilities of each of the four polypeptides were too close to be resolved. MALDI-TOF mass spectrometry (not shown) revealed a single peak of 156,434 Da, which is within 99.5% of the calculated mass (157,319 Da) of TF2.

The functionality of TF2 was determined by BIACORE® assay. TF2, C-DDD1-hMN-14+h679-AD1 (used as a control sample of noncovalent a_2b complex), or C-DDD2-hMN-14+ h679-AD2 (used as a control sample of unreduced a_2 and b components) were diluted to 1 µg/ml (total protein) and passed over a sensorchip immobilized with HSG. The response for TF2 was approximately two-fold that of the two control samples, indicating that only the h679-Fab-AD component in the control samples would bind to and remain on the sensorchip. Subsequent injections of WI2 IgG, an anti-idiotype antibody for hMN-14, demonstrated that only TF2 had a DDD-Fab-hMN-14 component that was tightly associated with h679-Fab-AD as indicated by an additional signal response. The additional increase of response units resulting from the binding of WI2 to TF2 immobilized on the sensorchip corresponded to two fully functional binding sites, each contributed by one subunit of C-DDD2-Fab-hMN-14. This was confirmed by the ability of TF2 to bind two Fab fragments of WI2 (not shown).

Example 2

C_{H3}-AD2-IgG Expression Vectors

A plasmid shuttle vector was produced to facilitate the conversion of any IgG-pdHL2 vector into a C_{H3}-AD2-IgG-pdHL2 vector. The gene for the Fc (C_{H2} and C_{H3} domains) was amplified by PCR using the pdHL2 vector as a template and the following oligonucleotide primers:

Fc BglII Left

```
AGATCTGGCGCACCTGAACTCCTG       (SEQ ID NO: 8)
```

Fc Bam-EcoRI Right

```
GAATTCGGATCCTTTACCCGGAGACAGGGAGAG. (SEQ ID NO: 9)
```

The amplimer was cloned in the pGemT PCR cloning vector (Promega). The Fc insert fragment was excised from pGemT with Xba I and Bam HI and ligated with AD2-pdHL2 vector that was prepared by digesting h679-Fab-AD2-pdHL2 (Rossi et al., Proc Natl Acad Sci USA 2006, 103:6841-6) with Xba I and Bam HI, to generate the shuttle vector Fc-AD2-pdHL2. To convert IgG-pdHL2 expression vectors to a C_{H3}-AD2-IgG-pdHL2 expression vectors, an 861 bp BsrG I/Nde I restriction fragment was excised from the former and replaced with a 952 bp BsrG I/Nde I restriction fragment excised from the Fc-AD2-pdHL2 vector. The following is a partial list of C_{H3}-AD2-IgG-pdHL2 expression vectors that have been generated and used for the production of recombinant humanized IgG-AD2 modules:

C_{H3}-AD2-IgG-hA20 (anti-CD20)
C_{H3}-AD2-IgG-hLL2 (anti-CD22)
C_{H3}-AD2-IgG-hL243 (anti-HLA-DR)
C_{H3}-AD2-IgG-hLL1 (anti-CD74)
C_{H3}-AD2-IgG-hR1 (anti-IGF-1R)
C_{H3}-AD2-IgG-h734 (anti-Indium-DTPA).

Example 3

Production of C_{H3}-AD2-IgG

Transfection and Selection of Stable C_{H3}-AD2-IgG Secreting Cell Lines

All cell lines were grown in Hybridoma SFM (Invitrogen, Carlsbad Calif.). C_{H3}-AD2-IgG-pdHL2 vectors (30 µg) were linearized by digestion with Sal I restriction endonuclease and transfected into Sp2/0-Ag14 (2.8×10^6 cells) by electroporation (450 volts, 25 µF). The pdHL2 vector contains the gene for dihydrofolate reductase allowing clonal selection as well as gene amplification with methotrexate (MTX).

Following transfection, the cells were plated in 96-well plates and transgenic clones were selected in media containing 0.2 µM MTX. Clones were screened for C_{H3}-AD2-IgG productivity by a sandwich ELISA using 96-well microtitre plates coated with specific anti-idiotype MAbs. Conditioned media from the putative clones were transferred to the micro-plate wells and detection of the fusion protein was accomplished with horseradish peroxidase-conjugated goat anti-human IgG F(ab')$_2$ (Jackson ImmunoResearch Laboratories, West Grove, Pa.). Wells giving the highest signal were expanded and ultimately used for production.

Production and Purification of C_{H3}-AD2-IgG Modules

For production of the fusion proteins, roller bottle cultures were seeded at 2×10^5 cells/ml and incubated in a roller bottle incubator at 37° C. under 5% CO_2 until the cell viability dropped below 25% (~10 days). Culture broth was clarified by centrifugation, filtered, and concentrated up to 50-fold by ultrafiltration. For purification of C_{H3}-AD2-IgG modules, concentrated supernatant fluid was loaded onto a Protein-A (MAB Select) affinity column. The column was washed to baseline with PBS and the fusion proteins were eluted with 0.1 M Glycine, pH 2.5.

Example 4

Generation of DDD2-mCD20(136-178) and Construction of DDD2-mCD20(136-178)-pdHL2

DDD2-mCD20(136-178)-pdHL2 is the expression vector for DDD2-mCD20(136-178), which comprises DDD2-linker-mCD20(136-178)-HHHHHH(HHHHHH disclosed as SEQ ID NO:30). The extracellular domain of mouse CD20 (mCD20) is referred to as mCD20(136-178), comprising amino acid residues 136 to 178 of the sequence shown below:

```
                                    (SEQ ID NO: 18)
  TLSHFLKMRRLELIQTSKPYVDIYDCEPSNSSEKNSPSTQYCN
```

The amino acid sequence of mouse CD20 xenoantigen is shown below.

```
                                    (SEQ ID NO: 7)
MSGPFPAEPTKGPLAMQPAPKVNLKRTSSLVGPTQSFFMRESKALGAVQ

IMNGLFHITLGGLLMIPTGVFAPICLSVWYPLWGGIMYIISGSLLAAAA

EKTSRKSLVKAKVIMSSLSLFAAISGIILSIMDILNMTLSHFLKMRRLE

LIQTSKPYVDIYDCEPSNSSEKNSPSTQYCNSIQSVFLGILSAMLISAF

FQKLVTAGIVENEWKRMCTRSKSNVVLLSAGEKNEQTIKMKEEIIELSG

VSSQPKNEEEIEIIPVQEEEEEEAEINFPAPPQEQESLPVENEIAP
```

The DNA segment comprising the nucleotide sequence of mCD20(136-178) flanked by BamHI and XhoI restriction sites is obtained by PCR using a full length murine CD20 cDNA clone as template and the two primers shown below:

Upstream Primer: BamHI_mCD20 Primer (30-mer)

```
                                    (SEQ ID NO: 31)
    5'-GGATCCACACTTTCTCATTTTTTAAAAATG
```

Downstream Primer: XhoI mCD20 Primer (30-mer)

```
5'-CTCGAGGTTACAGTACTGTGTAGATGGGGA  (SEQ ID NO: 32)
```

The PCR amplimer (141 bp) is cloned into the PGEMT® vector (PROMEGA®). A DDD2-pdHL2 mammalian expression vector, for example, N-DDD2-hG-CSF-His-pdHL2, is prepared for ligation with the amplimer by digestion with XbaI and Bam HI restriction endonucleases. The mCD20-amplimer is excised from PGEMT® with XbaI and Bam HI and ligated into the DDD2-pdHL2 vector to generate the expression vector DDD2-mCD20(136-178)-pdHL2.

Transfection and Screen to Obtain Clones Expressing DDD2-mCD20(136-178)

The vector DDD2-mCD20(136-178) is linearized by digestion with SalI enzyme and stably transfected into SpESF myeloma cells by electroporation (see, e.g., U.S. Pat. No. 7,537,930, the Examples section of which is incorporated herein by reference). A number of clones are found to have detectable levels of DDD2-mCD20(136-178) by ELISA, from which the best producing clone is selected and subsequently amplified with increasing methotrexate (MTX) concentrations from 0.1 to 0.8 μM over five weeks. At this stage, it is sub-cloned by limiting dilution and the highest producing sub-clone is expanded.

The clone is expanded to 34 roller bottles containing a total of 20 L of serum-free Hybridoma SFM with 0.8 μM MTX and allowed to reach terminal culture. The supernatant fluid is clarified by centrifugation and filtered (0.2 μM). The filtrate is diafiltered into 1× Binding buffer (10 mM imidazole, 0.5 M NaCl, 50 mM NaH$_2$PO$_4$, pH 7.5) and concentrated to 310 mL in preparation for purification by immobilized metal affinity chromatography (IMAC). The concentrate is loaded onto a 30-mL Ni-NTA column, which is washed with 500 mL of 0.02% Tween 20 in 1× binding buffer and then 290 mL of 30 mM imidazole, 0.02% Tween 20, 0.5 M NaCl, 50 mM NaH$_2$PO$_4$, pH 7.5. The product is eluted with 110 mL of 250 mM imidazole, 0.02% Tween 20, 150 mM NaCl, 50 mM NaH$_2$PO$_4$, pH 7.5. The purity of DDD2-mCD20(136-178) is assessed by SDS-PAGE under reducing conditions.

Example 5

Generation of 74-mCD20 DNL Vaccine Comprising hLL1 IgG Linked to Four Copies of mCD20(136-178)

C$_{H3}$-AD2-IgG-hLL1 (anti-CD74) is produced as described in Examples 2 and 3. The construct comprises an AD2 moiety attached to the C-terminal end of each heavy chain of the hLL1 IgG. DDD2-mCD20(136-178) is produced as described in Example 4. A DNL reaction is performed by mixing hLL1 IgG-AD2 and DDD2-mCD20 (136-178) in PBS containing 1 mM reduced glutathione. On the next day oxidized glutathione is added to a final con-

centration of 2 mM and the reaction mixture is purified on a Protein A column 24 h later. In this embodiment, two copies of the DDD2-mCD20 are attached to each AD2 moiety, resulting in a DNL complex comprising one hLL1 IgG moiety and four mCD20 xenoantigen moieties.

In an alternative embodiment, the Fab of hLL1 is linked to DDD2 and the mCD20(136-178) to AD2. Formation of a DNL construct as described above results in the formation of an MM vaccine, designated hLL1-F(ab)$_2$-mCD20(136-178), which comprises a single mCD20(136-178) attached to two Fab moieties of hLL1. The generation of AD2-mCD20(136-178) is described in Example 6.

Administration of 74-mCD20(136-178) or hLL1-F(ab)$_2$-mCD20(136-178) to subjects with MM induces an immune response against CD138^{neg}CD20$^+$ putative MM stem cells. The immune response is effective to reduce or eliminate MM disease cells in the subjects.

Example 6

Generation of Recombinant AD2-mCD20(136-178)

AD2-mCD20(136-178)-pdHL2 is the expression vector for recombinant AD2-mCD20(136-178), which comprises AD2-linker-mCD20(136-178)-HHHHHH(HHHHHH disclosed as SEQ ID NO:30). The DNA segment comprising the nucleotide sequence of mCD20(136-178) flanked by Bgl2 and Eag1 restriction sites is obtained by PCR using a full length murine CD20 cDNA clone as template and the two primers shown below:

Upstream Primer: Bgl2_mCD20 Primer (30-mer)

```
5'-AGATCTACACTTTCTCATTTTTTAAAAATG  (SEQ ID NO: 33)
```

Downstream Primer: Eag1_mCD20 Primer (48-mer)

```
                                     (SEQ ID NO: 34)
5' CGGCCGTCAGTGGTGGTGGTGGTGGTGGTGGTTACAGTACTGTGTAGATGG
```

The PCR amplimer (162 bp) is cloned into the PGEMT® vector (PROMEGA®). An AD2-pdHL2 mammalian expression vector, for example, N-AD2-hTransferrin-His-pdHL2, is prepared for ligation with the amplimer by digestion with Bgl2 and Eag1 restriction endonucleases. The mCD20-amplimer is excised from PGEMT® with Bgl2 and Eag1 and ligated into the AD2-pdHL2 vector to generate the expression vector AD2-mCD20(136-178)-pdHL2. Clones expressing AD2-mCD20(136-178) are obtained as described in Example 4 and AD2-mCD20(136-178) is purified from culture supernatants using Ni-select.

Example 7

AD and DDD Sequence Variants

In certain preferred embodiments, the AD and DDD sequences incorporated into the DNL complexes comprise the amino acid sequences of AD2 (SEQ ID NO:13) and DDD2 (SEQ ID NO:11), as described above. However, in alternative embodiments sequence variants of the AD and/or DDD moieties may be utilized in construction of the cytokine-MAb DNL complexes. The structure-function relationships of the AD and DDD domains have been the subject of investigation. (See, e.g., Burns-Hamuro et al., 2005, Protein Sci 14:2982-92; Carr et al., 2001, J Biol Chem 276:17332-38; Alto et al., 2003, Proc Natl Acad Sci USA

100:4445-50; Hundsrucker et al., 2006, Biochem J 396:297-306; Stokka et al., 2006, Biochem J 400:493-99; Gold et al., 2006, Mol Cell 24:383-95; Kinderman et al., 2006, Mol Cell 24:397-408.)

For example, Kinderman et al. (2006) examined the crystal structure of the AD-DDD binding interaction and concluded that the human DDD sequence contained a number of conserved amino acid residues that were important in either dimer formation or AKAP binding, underlined in SEQ ID NO:35 below. (See FIG. 1 of Kinderman et al., 2006.) The skilled artisan will realize that in designing sequence variants of the DDD sequence, one would desirably avoid changing any of the underlined residues, while conservative amino acid substitutions might be made for residues that are less critical for dimerization and AKAP binding.

Human DDD Sequence from Protein Kinase A

```
                                    (SEQ ID NO: 35)
SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA
```

Alto et al. (2003) performed a bioinformatic analysis of the AD sequence of various AKAP proteins to design an RII selective AD sequence called AKAP-IS (SEQ ID NO:12), with a binding constant for DDD of 0.4 nM. The AKAP-IS sequence was designed as a peptide antagonist of AKAP binding to PKA. Residues in the AKAP-IS sequence where substitutions tended to decrease binding to DDD are underlined in SEQ ID NO:12 below.

AKAP-IS Sequence

```
    QIEYLAKQIVDNAIQQA      (SEQ ID NO: 12)
```

Similarly, Gold (2006) utilized crystallography and peptide screening to develop a SuperAKAP-IS sequence (SEQ ID NO:19), exhibiting a five order of magnitude higher selectivity for the RII isoform of PKA compared with the RI isoform. Underlined residues indicate the positions of amino acid substitutions, relative to the AKAP-IS sequence, that increased binding to the DDD moiety of RIIα. In this sequence, the N-terminal Q residue is numbered as residue number 4 and the C-terminal A residue is residue number 20. Residues where substitutions could be made to affect the affinity for RIIα were residues 8, 11, 15, 16, 18, 19 and 20 (Gold et al., 2006). It is contemplated that in certain alternative embodiments, the SuperAKAP-IS sequence may be substituted for the AKAP-IS AD moiety sequence to prepare cytokine-MAb DNL constructs. Other alternative sequences that might be substituted for the AKAP-IS AD sequence are shown in SEQ ID NO:20-22. Substitutions relative to the AKAP-IS sequence are underlined. It is anticipated that, as with the AKAP-IS sequence shown in SEQ ID NO:19, the AD moiety may also include the additional N-terminal residues cysteine and glycine and C-terminal residues glycine and cysteine.

SuperAKAP-IS

```
    QIEYVAKQIVDYAIHQA      (SEQ ID NO: 19)
```

Alternative AKAP Sequences

```
    QIEYKAKQIVDHAIHQA      (SEQ ID NO: 20)

    QIEYHAKQIVDHAIHQA      (SEQ ID NO: 21)

    QIEYVAKQIVDHAIHQA      (SEQ ID NO: 22)
```

Stokka et al. (2006) also developed peptide competitors of AKAP binding to PKA, shown in SEQ ID NO:23-25. The peptide antagonists were designated as Ht31 (SEQ ID NO:23), RIAD (SEQ ID NO:24) and PV-38 (SEQ ID NO:25). The Ht-31 peptide exhibited a greater affinity for the RII isoform of PKA, while the RIAD and PV-38 showed higher affinity for RI.

Ht31

```
    DLIEEAASRIVDAVIEQVKAAGAY      (SEQ ID NO: 23)
```

RIAD

```
    LEQYANQLADQIIKEATE      (SEQ ID NO: 24)
```

PV-38

```
    FEELAWKIAKMIWSDVFQQC      (SEQ ID NO: 25)
```

Hundsrucker et al. (2006) developed still other peptide competitors for AKAP binding to PKA, with a binding constant as low as 0.4 nM to the DDD of the RII form of PKA. The sequences of various AKAP antagonistic peptides is provided in Table 1 of Hundsrucker et al. (incorporated herein by reference). Residues that were highly conserved among the AD domains of different AKAP proteins are indicated below by underlining with reference to the AKAP IS sequence (SEQ ID NO:12). The residues are the same as observed by Alto et al. (2003), with the addition of the C-terminal alanine residue. (See FIG. 4 of Hundsrucker et al. (2006), incorporated herein by reference.) The sequences of peptide antagonists with particularly high affinities for the RII DDD sequence are shown in SEQ ID NO:26-28.

AKAP-IS

```
    QIEYLAKQIVDNAIQQA      (SEQ ID NO: 12)
```

AKAP7δ-wt-pep

```
    PEDAELVRLSKRLVENAVLKAVQQY      (SEQ ID NO: 26)
```

AKAP7δ-L304T-pep

```
    PEDAELVRTSKRLVENAVLKAVQQY      (SEQ ID NO: 27)
```

AKAP7δ-L308D-pep

```
    PEDAELVRLSKRDVENAVLKAVQQY      (SEQ ID NO: 28)
```

Carr et al. (2001) examined the degree of sequence homology between different AKAP-binding DDD sequences from human and non-human proteins and identified residues in the DDD sequences that appeared to be the most highly conserved among different DDD moieties. These are indicated below by underlining with reference to the human PKA RIIα DDD sequence of SEQ ID NO:35. Residues that were particularly conserved are further indicated by italics. The residues overlap with, but are not identical to those suggested by Kinderman et al. (2006) to be important for binding to AKAP proteins.

```
                                    (SEQ ID NO: 35)
SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA
```

The skilled artisan will realize that in general, those amino acid residues that are highly conserved in the DDD and AD sequences from different proteins are ones that it may be preferred to remain constant in making amino acid substitutions, while residues that are less highly conserved may be more easily varied to produce sequence variants of the AD and/or DDD sequences described herein.

The skilled artisan will realize that these and other amino acid substitutions in the antibody moiety or linker portions of the DNL constructs may be utilized to enhance the therapeutic and/or pharmacokinetic properties of the resulting DNL constructs.

Example 8

Effects of hLL1 on DCs—Efficient Binding of hLL1 with Different Subsets of APCs

Early studies demonstrated that CD74 is expressed in most antigen-presenting cells including blood DCs, B cells, monocytes. To further characterize the expression profile of CD74 in APCs, we examined the expression of CD74 in different subsets of human PBMCs and in vitro monocyte-derived DCs. Using the gating strategy that is shown in FIG. 1A, we found all of the blood DC subsets, the myeloid DC1 (MDC1) and DC2 (MDC2), and plasmacytoid DC (PDC) expressed CD74, with MDC2 expressing the highest level of CD74 (FIG. 1B). CD74 was also expressed in monocyte-derived immature DCs at much higher level than in LPS-matured DCs (FIG. 2A). Consistent with the CD74 expression profiles, hLL1 bound efficiently with blood DC subsets, B cells, monocytes, and monocyte-derived immature DCs (FIG. 1C, FIG. 2B), but not LPS-matured DCs (FIG. 2B, FIG. 2C). The binding efficiency of hLL1 in these APC subsets correlates well with their CD74 expression levels. These data provide the basis for in vivo targeting of antigen to APCs using hLL1 as the targeting vehicle by Dock-and-lock technology.

Cytotoxic Effect of hLL1 on CD74-Expressing Malignant B Cells but not on Normal DCs

Since CD74 is highly expressed in immature DCs, with which hLL1 binds efficiently, as shown in FIG. 1A and FIG. 1B, we wondered if hLL1 has the same cytotoxicity in DCs, as it does a in CD74-expressing B cell lymphoma, which was shown previously (Stein et al., Blood 2004, 104:3705-11). To this end, the effects of hLL1 on the cell viability of B cell malignancy Daudi cells and human monocyte-derived DCs were side-by-side compared using an MTS assay and microscope imaging. The results demonstrated that hLL1, in the presence of GAH (goat anti-human antibody), the second antibody for hLL1 cross-linking, significantly reduced cell viability of Daudi cells but not DCs (FIG. 3A), which normally expressed high level of CD74 as shown above. The microscopic imaging showed that Daudi cells treated with hLL1 crosslinked with GAH became clumped and condensed, while the DCs maintained normal morphology after the same treatment (FIG. 3C, FIG. 3D). The cytotoxicity against Daudi cells by hLL1 cross-linked with GAH was consistent with the earlier study by Stein et al. (2004) showing that hLL1 was cytotoxic to B cell malignancies in vitro and in vivo. The lack of cytotoxicity of hLL1 plus GAH on DCs was further demonstrated in apoptosis assay, which showed that the hypodiploid nuclei populations were not influenced by hLL1 cross-linked with GAH (not shown).

To further confirm the lack of cytotoxicity of hLL1 on DCs, we performed apoptosis assay using flow cytometry. The nuclei from hLL1 treated immature DCs were obtained and stained with PI for flow cytometry analysis. The PI+ particles were gated first, and the debris was excluded by gating out the SSC-low particles. The resulting gated nuclei were analyzed for apoptosis by measuring hypodiploid nuclei population (FIG. 2A). The results demonstrate that hLL1 had no influence on DC apoptosis in both donors (FIG. 2B, FIG. 2C), in the presence or absence of a second mAb (20 μg/ml) for cross-linking (GAH, F(ab')$_2$ GAH IgG Fey-specific). These data demonstrated that hLL1, unlike its cytotoxic effect on B cell malignancies, has little cytotoxicity against normal dendritic cells which also express CD74 surface antigen.

Moderate Enhancement of DC Constitutive Maturation by hLL1

Human IgG can interact with DCs through FcR ligation and has opposing effects on DC maturation depending on which subtype(s) of FcR is involved. hLL1, as a humanized IgG, may interact with human DCs not only through CD74 but also through FcR expressed on DCs. For this reason, we speculated that hLL1 may influence DC functions through interaction with CD74 or FcR, or both. To investigate this, we tested the effect of hLL1 on DC constitutive maturation during in vitro culture of monocytes in the presence of hGM-CSF and hIL-4.

Since DC maturation is usually reflected by its morphological change, we also examined if hLL1 treatment has any effect on DC morphology. As shown in FIG. 3B, DCs treated with hLL1, at different doses for various days, in the absence or presence of GAH cross-linking, appeared healthy and intact. The hLL1-treated DCs exhibited some minor morphological changes featured with fiber-like cells, which are similar to but less obvious than LPS-treated DCs (not shown).

As mature DCs differ from immature DCs mainly in the upregulation of antigen-presenting and costimulatory molecule expression, altered cytokine production, and enhanced T-cell stimulatory ability, we then investigated if hLL1 has any effect on the expression level of antigen-presenting molecule HLA-DR and costimulatory molecules CD54 and CD86 in DCs (FIG. 4). The results show that hLL1 could upregulate HLA-DR, CD54, and CD86 in a dose-dependent manner within the range of hLL1 concentrations at 0.05-5 ug/ml (FIG. 4A). However, the effect was not strong, as the expression of HLA-DR and costimulatory molecules, CD54 and CD86, were only 10% upregulated at 5 μg/ml hLL1 compared to 0 ug/ml (FIG. 4B). At the highest concentration (50 μg/ml), the expression of HLA-DR, CD54 and CD86 was not further upregulated but slightly reduced, compared to hLL1 at 5 μg/ml (FIG. 4B). These results indicate that hLL1, although not potently, could enhance the constitutive maturation of DCs.

No Significant Influence on T Cell Expansion by hLL1-Treated DCs

The functional difference between immature DCs and mature DCs is that mature DCs have a stronger capacity to stimulate T cell proliferation and expansion. Since hLL1 could enhance the constitutive maturation by upregulating the expression of HLA-DR, CD54 and CD86 expression in DCs (FIG. 4B), we determined whether this DC-maturing effect could be reflected by an enhanced T cell expansion by DCs. As shown in FIG. 5, DCs treated with hLL1 at 0.05 to 50 μg/ml did not influence the DC-mediated T cell expansion, including total T cells, CD4+ and CD4− T cells (FIG. 5). This result suggests that hLL1-enhanced DC constitutive maturation was not strong enough to be translated into an enhanced T cell stimulatory ability.

Polarization of Naïve CD4+ T Cells Toward Th1 Effector Cells by hLL1-Treated DCs

However, DCs have another important function: the polarization of naïve CD4 T cells to differentiate into different effector cells, Th1, Th2, Th17, as well as newly defined Th17-1 cells. Th1 cells are critical for cellular immunity against intracellular pathogens and cancers, whereas induction of Th2 cells is responsible for humoral immunity. The IL-17-producing Th17 and Th17-1 cells are other polarized cell populations which have multiple functions in immunity to certain pathogens and autoimmune inflammation. The polarization of these effector cells is largely mediated through DC-secreted cytokines, the so-called "signal 3", that DCs provide to T cells in the DC/T cell synapse. The CD4+ naïve T cells can differentiate into Th1, Th2 and Th0 cells which mediate different effector functions, among which the Th1 effector cells play an essential role in maintaining CTL response against cancer and infectious diseases. We have shown that hLL1 at 0.05 to 50 µg/ml could enhance DC constitutive maturation in a weak but dose-dependent manner, but DCs treated with these concentrations of hLL1 didn't influence the DC-mediated T cell expansion (FIG. 5). We were then interested if the hLL1-treated DCs could influence the polarization of CD4+ naïve T cells. As shown in FIG. 5, hLL1-treated DCs polarized the CD4+ naïve T cells to differentiate toward more Th1 effector cells and fewer Th2 and Tnp cells. These results indicate that DCs can be functionally modulated by hLL1. As Th1 plays a crucial role in adaptive immunity against tumor and infectious diseases, hLL1 may have an adjuvant-like activity when used in vaccination.

Example 9

In Vitro Properties of 74-mCD20—Induction of hCD20-Specific Immunity by 74-mCD20 in Human PBMCs

CD20 is a self antigen normally expressed on B cells, which is theoretically difficult to target by vaccine strategies due to immune tolerance. However, specific T-cell immune response to CD20 has been achieved in tumor bearing mice by vaccination with a minigene encoding the extracellular domain of human CD20 (Palomba et al., Clin Cancer Res 2005; 11:370-9), or a conjugate comprising the extracellular domain of human CD20 and a carrier protein with QS21 adjuvant (Roberts et al., Blood 2002; 99:3748-55). Several other reports have also demonstrated the feasibility of using xenoantigens to break immune tolerance, as shown for MUC1 in animal models (Ding et al., Blood 2008; 112: 2817-25; Soares et al., J Immunol 2001; 166:6555-63) as well as in patients (Ramanathan et al., Cancer Immunol Immunother 2005; 54:254-64). To test whether 74-mCD20 could successfully induce hCD20-specific immunity and overcome the immune tolerance of CD20, the following experiment is performed.

Human DCs are generated from PBMCs by culturing for 5 days in the presence of hGM-CSF and hIL-4. The immature DCs are loaded with 74-mCD20, and matured by LPS plus IFN-gamma. The mature DCs are used to stimulate autologous PBMCs for 10 days. Restimulation with the same loaded DCs is performed twice weekly. After the last restimulation, the T cells are tested for their antigen specificity by measuring cytokine response (IFN-gamma) upon stimulation by sorted CD20-positive MM cancer stem cells. The CD20-negative MM cells are used as a control. The T

cells show a positive reaction to CD20-positive MM cancer stem cells but not to control CD20-negative MM cells.

Specific Binding, Internalization and Intracellular Location of 74-m CD20 in Various Antigen Presenting Cells In Vitro

Our preliminary data have shown that hLL1 efficiently and specifically binds with different APCs, including myeloid DC1 and myeloid DC2, plasmacytoid DC, B cells and monocytes. In order to confirm that 74-mCD20 has the same efficiency and specificity in binding with APCs as hLL1 alone, the following experiment is performed.

74-mCD20 and the control M1-mCD20 (comprising the anti-MUC1 antibody hPAM4 linked to four copies of mCD20) are used. Binding assays are performed as follows. Briefly, 15 mg of 74-mCD20 or M1-mCD20 are labeled with a ZENON™ ALEXA FLUOR® 488 human IgG labeling kit (INVITROGEN®) following the manufacturer's instructions. The labeled preparations are used to stain the human PBMCs as described below.

Human PBMCs isolated from buffy coat using FICOLL-PAQUE™ are treated with human FcR blocking Reagent (Miltenyi Biotec, 1:20 dilution) at 4° C. for 10 min. The washed cells are stained with specifically labeled mAbs and analyzed by flow cytometry (FACSCALIBUR®). The labeled mAbs used for the study include FITC-labeled anti-CD74 mAb ALEXA FLUOR® 488-labeled 74-mCD20; ALEXA FLUOR® 488-labeled M1-mCD20; PE-conjugated anti-CD19 mAb (for B cells); PE-conjugated anti-CD14 mAb (for monocytes); and APC-conjugated mAb to BDCA-1 (for MDC1), BDCA-2 (for PDC), or BDCA-3 (for MDC2). A gating strategy is used for identification of B cells, monocytes, MDC1, MDC2, and PDC. Data were analyzed by FlowJo software for mean fluorescence intensity and positive cell populations expressing the surface markers.

To see if 74-mCD20 is internalized to endosomes for further processing to MHC class II presentation and MHC class I cross-presentation, the following experiment is performed. 74-mCD20 or M1-mCD20 is mixed with human PBMCs, and incubated at 4° C. for 1 hr, followed by extensive washing. The cells are then transferred to 37° C., fixed at different time points (0, 15, 30, or 45 min) and stained with ALEXA FLUOR®-labeled anti-human IgG secondary antibody with or without prior permeabilization. The mean fluorescence is determined by flow cytometry, and the amount of internalized antibody is calculated by subtracting the mean fluorescence in fixed cells (surface bound) from that recorded with fixed and permeabilized cells (internalized and surface bound) at various time points.

The results show that the 74-mCD20 DNL complex has the same efficiency and specificity in binding with APCs as hLL1 alone.

Example 10

Induction of hCD20-Specific Immune Responses by 74-mCD20 In Vivo

Intrahepatic injection of CD34+ human cord blood cells (HLA A1 healthy donor) into irradiated newborn Rag2−/−γc−/− mice is performed to generate the animal model for a reconstituted human adaptive immune system including human T, B, and DC cells, and structured primary and secondary lymphoid organs (Huff et al., J Clin Oncol. 2008, 26:2895-900; Yang and Chang, Cancer Invest. 2008, 26:741-55). These mice are called Hu-Rag2−/−γc−/− mice.

To assess the immune responses induced by 74-mCD20, human CD34+ cells reconstituted in Rag2–/–γc–/– mice are immunized weekly for three times with 74-mCD20 or M1-mCD20(50 µg per mouse), in combination with or without CpG (50 µg per mouse) for in vivo DC maturation. Five days after the last immunization, splenocytes of each animal are isolated and restimulated with HLA-matched MM cancer stem cells for cytokine (IFN-gamma) production, as assessed by intracellular cytokine staining with flow cytometry. The specific cytotoxicity against MM cancer stem cells is assessed by a calcein AM release assay with MM cancer stem cells as the target cells. The CD20+ MM cancer stem cells are isolated from the MM cell line RPMI18226 using magnetic beads. The stem cell property is verified by staining with aldehyde dehydrogenase. The results indicate that 74-mCD20 is capable of inducing an anti-hcd20 specific immune response in vivo.

Example 11

Therapeutic Potential of 74-mCD20 Against MM Cancer Stem Cells: In Vivo Evaluation by hPBMC/NOD/SCID Mouse Model or Adoptive Transfer

The best way for in vivo evaluation of the therapeutic effect of 74-mCD20 is to immunize an animal model that can support both the growth of MM and the development of a human adaptive immune system. Since human CD34+ cell-reconstituted Rag2–/–γc–/– mice are immune-competent, which may not support MM growth, the hPBMC/NOD/SCID mouse model is used to test the therapeutic effect of 74-mCD20 against MM stem cells. The NOD/SCID mice have been used for engraftment of clonogenic multiple myeloma stem cells by Matsui et al. (Blood 2004, 103:2332-6; Cancer Res 2008, 68:190-7).

The NOD/SCID mice are also used for evaluating the therapeutic effect by co-engraftment of tumor cells and hPBMC. By carefully adjusting the cell numbers infused, this model can support both tumor growth and hPBMC engraftment, and has been used for testing the effect of an in vivo vaccine targeting DC-SIGN.

Four to six-week-old female NOD/SCID mice (Jackson Laboratories, Barr Harbor, Me.) are irradiated with 300 cGy (84 cGy/min using a 137Cs gamma irradiator). 12-16 h later, sorted CD20+ MM cancer stem cells (2 million) are injected via dorsal tail vein. Meanwhile, a mixture of human PBMCs (3 million), immature DC (30,000) and the DNL vaccine is injected into the mice subcutaneously. At certain time points (days), mice are euthanatized and bone marrow is harvested from the long bones and the engraftment and therapeutic efficacy are determined by staining for human CD138+ MM cells.

In order to further evaluate the therapeutic potential of 74-mCD20, an alternative method by adoptive transfer is used to test the vaccine-elicited cytotoxicity against MM stem cells. The human CD34+ cell-reconstituted Rag2–/–γc–/– mice are immunized with 74-mCD20 as described above. The splenocytes are harvested and injected via the tail vein into NOD/SCID mice engrafted with CD20+ MM cancer stem cells. At certain time points (days), mice are euthanatized and bone marrow is harvested from the long bones and the engraftment and therapeutic efficacy are determined by staining for human CD138+ MM cells. The

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results confirm that 74-mCD20 is capable of inducing an immune response against CD20+ MM stem cells in vivo.

Example 12

Generation of DDD2-mPAP and DNL Vaccine Complex

A DDD2 conjugated PAP xenoantigen is generated from murine prostatic acid phosphatase according to the method of Example 4. The efficacy of dendritic cell based vaccination with a PAP xenoantigen has been previously disclosed (Fong et al. J Immunol 2001, 167:7150-56). A DDD2-mPAP-pdHL2 expression vector is constructed as described in Example 4 and the DDD2-mPAP xenoantigen fusion protein is expressed in cell culture according to Example 4. The murine prostatic acid phosphatase sequence is disclosed, for example, in the NCBI database at Accession No. AAF23171. A DDD2-mPAP-6H is fusion protein is expressed and purified by immobilized metal affinity chromatography (IMAC) as described in Example 4.

A DNL construct comprising one copy of C_{H3}-AD2-IgG-hLL1 (anti-CD74) and four copies of DDD2-mPAP is prepared according to the methods of Example 5. The hLL1 IgG moiety comprises an AD2 sequence attached to the C-terminal end of each heavy chain of the hLL1 IgG. A DNL reaction is performed by mixing hLL1 IgG-AD2 and DDD2-mPAP in PBS containing 1 mM reduced glutathione. On the next day oxidized glutathione is added to a final concentration of 2 mM and the reaction mixture is purified on a Protein A column 24 h later. Two copies of the DDD2-mPAP are attached to each AD2 moiety, resulting in a DNL complex comprising one hLL1 IgG moiety and four mPAP xenoantigen moieties.

Administration of DNL vaccine anti-CD74-mPAP to subjects with prostate cancer induces an immune response against PAP expressing prostatic cancer stem cells. The immune response is effective to reduce or eliminate prostatic cancer cells in the subjects.

Example 13

Generation of DDD2-mEGFR and DNL Vaccine Complex

A DDD2 conjugated EGFR xenoantigen is generated from murine EGFR according to the method of Example 4. The efficacy of EGFR xenoantigen at inducing a humoral immune response has been previously disclosed (Fang et al. Int J Mol Med 2009, 23:181-88). A DDD2-mEGFR-pdHL2 expression vector comprising the extracellular domain of murine EGFR is constructed as described in Example 4 and the DDD2-mEGFR xenoantigen fusion protein is expressed in cell culture according to Example 4. The murine EGFR sequence is disclosed, for example, in the NCBI database at Accession No. AAG43241. A DDD2-mEGFR-6H is fusion protein is expressed and purified by immobilized metal affinity chromatography (IMAC) as described in Example 4.

A DNL construct comprising one copy of C_{H3}-AD2-IgG-hLL1 (anti-CD74) and four copies of DDD2-mEGFR is prepared according to the methods of Example 5. The hLL1 IgG moiety comprises an AD2 sequence attached to the C-terminal end of each heavy chain of the hLL1 IgG. A DNL reaction is performed by mixing hLL1 IgG-AD2 and DDD2-mEGFR in PBS containing 1 mM reduced glutathione. On the next day oxidized glutathione is added to a final concentration of 2 mM and the reaction mixture is purified on

a Protein A column 24 h later. Two copies of the DDD2-mEGFR are attached to each AD2 moiety, resulting in a DNL complex comprising one hLL1 IgG moiety and four mEGFR xenoantigen moieties.

Administration of DNL vaccine anti-CD74-mEGFR to subjects with EGFR-expressing NSCLC induces an immune response against EGFR-expressing cancer stem cells. The immune response is effective to reduce or eliminate EGFR positive cancer cells in the subjects.

The skilled artisan will realize that DNL-based vaccines incorporating xenoantigen moieties corresponding to a wide variety of tumor-associated antigens may be constructed and utilized according to the techniques described herein.

All of the COMPOSITIONS and METHODS disclosed and claimed herein can be made and used without undue experimentation in light of the present disclosure. While the compositions and methods have been described in terms of preferred embodiments, it is apparent to those of skill in the art that variations may be applied to the COMPOSITIONS and METHODS and in the steps or in the sequence of steps of the METHODS described herein without departing from the concept, spirit and scope of the invention. More specifically, certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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        35                  40                  45

Gln Ile Met Asn Gly Leu Phe His Ile Thr Leu Gly Gly Leu Leu Met
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65                  70                  75                  80

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Cys Glu Pro Ser Asn Ser Ser Glu Lys Asn Ser Pro Thr Gln Tyr
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Cys Asn Ser Ile Gln Ser Val Phe Leu Gly Ile Leu Ser Ala Met Leu
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Ile Ser Ala Phe Phe Gln Lys Leu Val Thr Ala Gly Ile Val Glu Asn
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        35                  40                  45

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1   5   10   15

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<400> SEQUENCE: 28

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1   5   10   15

Ala Val Leu Lys Ala Val Gln Gln Tyr
            20   25

<210> SEQ ID NO 29
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<400> SEQUENCE: 29

Pro Lys Ser Cys
1

<210> SEQ ID NO 30
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<400> SEQUENCE: 30

His His His His His His
1   5

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-continued

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<400> SEQUENCE: 33

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<400> SEQUENCE: 34

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<210> SEQ ID NO 35
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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 35

Ser His Ile Gln Ile Pro Pro Gly Leu Thr Glu Leu Leu Gln Gly Tyr
1               5                   10                  15

Thr Val Glu Val Leu Arg Gln Gln Pro Pro Asp Leu Val Glu Phe Ala
            20                  25                  30

Val Glu Tyr Phe Thr Arg Leu Arg Glu Ala Arg Ala
            35                  40
```

What is claimed is:

1. A method of inducing an immune response against a xenoantigen moiety comprising:

a) obtaining a DNL complex comprising; i) a chimeric, humanized or human anti-CD74 antibody moiety that binds to human CD74, wherein the antibody moiety is attached to an AD (anchor domain) moiety, wherein the AD moiety has an amino acid sequence selected from the group consisting of SEQ ID NO:13; SEQ ID NO:12; SEQ ID NO:19; SEQ ID NO:20; SEQ ID NO:21; SEQ ID NO:22; SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:25; SEQ ID NO:26; SEQ ID NO:27 and SEQ ID NO:28; and ii) a mouse xenoantigen moiety selected from the group consisting of carbonic anhydrase IX, alpha-fetoprotein (AFP), α-actinin-4, CD1, CD3, CD4, CD5, CD8, CD11A, CD14, CD15, CD16, CD18, CD19, CD20, CD21, CD22, CD23, CD25, CD29, CD30, CD33, CD37, CD38, CD40, CD40L, CD45, CD46, CD52, CD54, CD55, CD59, CD64, CD74, CD79a, CD80, CD83, CD95, CD126, CD138, CD147, CD154, PSMA, fibronectin, folate receptor, IL-6R, IL-13R, IL-17R, IL-18R, IL-6, IL-8, IL-12, IL-15, IL-17, IL-18, IFN-γ, IFN-α, IFN-β, macrophage migration inhibitory factor (MIF), mCRP, MIP-1B, MUC1, MUC2, MUC4, placental growth factor, p53, prostatic acid phosphatase, RANTES, tenascin, and TNF-α, wherein the xenoantigen moiety is attached to a DDD (dimerization and docking domain)

moiety, wherein the amino acid sequence of the DDD moiety is residues 1-44 of human protein kinase A (PKA) RIIα and wherein two copies of the DDD moiety form a dimer that binds to the AD moiety to form the DNL complex; and

b) administering the complex to a human subject to induce an immune response against the xenoantigen.

2. The method of claim **1**, wherein the antibody moiety comprises two heavy chains and each heavy chain is attached at its C-terminal end to an AD moiety and the complex comprises one antibody moiety and four xenoantigen moieties.

3. The method of claim **1**, wherein the xenoantigen is CD20.

4. The method of claim **1**, wherein the amino acid sequence of the DDD moiety is selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:10.

5. The method of claim **4**, wherein the amino acid sequence of the DDD moiety is SEQ ID NO:11.

6. The method of claim **1**, wherein the amino acid sequence of the AD moiety is SEQ ID NO:13.

7. The method of claim **1**, wherein the antibody moiety is a humanized or chimeric LL1 anti-CD74 antibody or antigen-binding fragment thereof comprising the light chain variable complementarity-determining region (CDR) sequences CDR1 (RSSQSLVHRNGNTYLH; SEQ ID NO:1), CDR2 (TVSNRFS; SEQ ID NO:2), and CDR3 (SQSSHVPPT; SEQ ID NO:3) and the heavy chain variable region CDR sequences CDR1 (NYGVN; SEQ ID NO:4), CDR2 (WINPNTGEPTFDDDFKG; SEQ ID NO:5), and CDR3 (SRGKNEAWFAY; SEQ ID NO:6).

8. The method of claim **3**, wherein the amino acid sequence of the CD20 xenoantigen moiety is SEQ ID NO:7.

9. The method of claim **1**, wherein the DNL complex is capable of inducing an immune response against $CD138^{neg}CD20^{+}$ MM stem cells.

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